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(54) Title: ENHANCED MERISTEMATIC ACTIVITY AND COMPETENCE BY OVEREXPRESSION OF TONOPLAST PY-  
ROPHOSPHATASE

(57) Abstract: A transgenic plant having increased meristematic activity and competence effectuating in larger leave, stem, flower,  
fruit and root structures comprising a polynucleotide sequence causing upregulated expression of vacuolar pyrophosphatase.

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## **BACKGROUND OF THE INVENTION**

### **1. Field of the Invention**

The present invention relates to genetically-altered plants that are hardy with respect to environmental stresses, such as drought and/or freezing, oversized with respect to vegetative and/or sexual structure (as compared to their normal phenotypic counterparts), and capable of growing in media of high salinity. Such plants also display high meristematic activity, and increased in cellular division activity.

### **2. Background of the Related Art**

The prospects for feeding humanity as we enter the new millennium are formidable. Given the every increasing world population, it remains a major goal of agricultural research to improve crop yield. It also is a major goal of horticultural research to develop non-crop plants which are hardier, such as ornamental plants, grasses, shrubs, and other plants found useful or pleasing to man.

Until recently crop and horticultural improvements depended on selective breeding of plants having desirable characteristics. Such selective breeding techniques, however, were often less than desirable as many plants have within them heterogenous genetic complements that do not result in identical desirable traits of their parents.

Advances in molecular biology have allowed mankind to manipulate the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants with increased pest resistance, plants that are capable of expressing pharmaceuticals and other chemicals, and plants that express beneficial traits. Advantageously such plants not only contain a gene of interest, but remain fertile.

One area of interest of late in plant sciences has been the development of plants with improved stress resistance. In general, plants possess and maintain adaptive

mechanisms to ensure survival during periods of adverse environmental conditions. Two  
common stresses that plants commonly encounter are freezing and drought, both of which  
are associated with cellular dehydration. It is known that certain plants contain genes  
turned on by exposure to cold or prolonged periods of dehydration that encode for  
5 products that are directly or indirectly responsible for providing greater resistance to  
drought and/or freeze than many of their counterparts. A number of genes responsive to  
heat and water stress have now been characterized (See, e.g., U.S. Pat. Nos. 5,837,545,  
5,071,962, 4,707,359). These genes are believed by many to produce certain proteins,  
such as "Water Stress Proteins", that are postulated to aid the plants survival. For  
10 example, certain plants exposed to stress conditions produce a hormone called abscisic acid  
(ABA) which helps plants close their stomata, thereby reducing the severity of the stress.  
Unfortunately, ABA is known to inhibit the formation of new leaves, to cause flowers and  
fruit to drop off, and to lead to a reduction in yield.

Most tropical plants are not believed to have evolved the ability to tolerate  
15 prolonged drought and/or freezing. Conversely, many temperate plants are known to have  
developed at least some ability to tolerate such conditions. The productivity of plant  
varieties in dry conditions, and after freeze, differ dramatically. For example, tobacco  
(*Nicotiana* spp.) produces fresh leaves that are highly sensitive to drought, and cannot be  
produced commercially in areas with a limited water supply and high degree of  
20 evaporation. In contrast to water stress, very little is known about proteins and genes  
which participate in freezing tolerance. However, it has been hypothesized that a major  
component of freeze tolerance may involve tolerance to dehydration (See, e.g., Yelenosky,  
G.C., Guy, L. (1989) *Plant Physiol.* 89: 444-451).

Another particular area of interest of late has been the development of  
25 plants with improved abilities to grow in salinized soil. Salinization of soil occurs when  
water supplies contain dissolved salt. Upon evaporation of water from such supplies, salts  
gradually accumulate in the soil. The progressive salinization of irrigated land  
compromises the future of agriculture in many of the most productive areas of our planet  
(Serrano, R., *et al.*, *Crit. Rev. Plant Sci.*, 13:121-138 (1994)). For example, arid regions  
30 offer optimal photoperiod and temperature conditions for the growth of most crops, but  
suboptimal rainfall. Artificial irrigation has solved the problem only in the short term as it  
has been found that soils in such environments frequently are rapidly salinized. To grow

in salinized environments, plants must maintain a much lower ratio of  $\text{Na}^+/\text{K}^+$  in their cytoplasm than that present in the soil, preventing the growth of a number of plants, including food crops.

Physiological studies suggest that salt exclusion in the root, and/or salt  
5 sequestration in the leaf cell vacuoles, are critical determinants for salt tolerance (Kirsch, M., *et al.*, *Plant Mol. Biol.*, 32:543-547 (1996)). Toxic concentrations of sodium chloride (NaCl) build up first in the fully expanded leaves where NaCl is compartmentalized in the vacuoles. Only after their loading capacity is surpassed, do the cytosolic and apoplasmic concentrations reach toxic levels, ultimately leading to loss of turgor, *ergo* plant death. It  
10 has been suggested that hyperacidification of the vacuolar lumen via the V-ATPase provides extra protons required for a  $\text{Na}^+/\text{H}^+$  exchange-activity leading to the detoxification of the cytosol (Tsiantis, M.S., *et al.*, *Plant J.*, 9:729-736 (1996)). Salt stress is known to increase both ATP- and pyrophosphate (PPi)-dependent  $\text{H}^+$  transport in tonoplast vesicles of, for example, sunflower seedling roots. Salt treatments also induce  
15 an amiloride-sensitive  $\text{Na}^+/\text{H}^+$  exchange activity (Ballesteros, E., *et al.*, *Physiologia Plantarum*, 99:328-334 (1997)). In the halophyte *Mesembryanthemum crystallinum*, high NaCl stimulates the activities of both the vacuolar  $\text{H}^+$ -ATPase (V-ATPase) and a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter in leaf cells.

Yet another area of agricultural interest is to improve the yield of crop  
20 plants and to improve the aesthetic qualities of certain decorative plants. The yield of a plant crop, and the aesthetics of certain decorative plants, may be improved by growing plants that are larger than the wild-type plant in vegetative and/or reproductive structure, as well as improving the growth rate of plants.

A number of compounds have been touted in the prior art as improving the  
25 rate of plant growth and biomass production in useful components of the plant. For example., cyclodextrins applied to tissue culture media has been asserted to improve the rate of cell tissue culture growth by mechanisms including increased cell division (See, e.g., U.S. Patent No. 6,087,176). Certain plant growth hormones, such as auxins (which promote, among other things, root growth), cytokinins, and gibberellic acid (which  
30 promotes, among other things, stem growth) when applied to plant tissues are also known to promote increased cellular division. It also is known in the art that certain growth

factors may be used to increase plant and/or plant flower size. Unfortunately, isolation and application of such growth hormones and factors is costly and time consuming.

U.S. Patent No. 5,859,338 discloses that modification of the CLAVATA1 gene of *Arabidopsis thaliana* causes a loss normal control of cell division in shoot apical meristems and floral meristems. In either case, the loss of control is said to cause an enlargement of the meristem. In flowers, the enlargement is said to lead to an increase in the number of floral organs, including an increase in carpel number, which increases fruit size and seed number. U.S. Patent No. 5,859,338 provides clavata1 nucleic acids and proteins, and modified clavata1 nucleic acids and proteins, to result in altered meristem phenotypes.

U.S. Patent No. 5,750,862 discloses a method for controlling plant cell growth comprising modulating the level and or catalytic activity of a cell cycle control protein in the plant. In particular the patent discloses that by elevating levels of the protein p34<sup>cdc2</sup>, regeneration into plants of single or groups of cells can be facilitated. Control of regeneration may also be effectuated by control of regulatory elements which indirectly result in modulation of p34<sup>cdc2</sup> activity.

A need, therefore, exists for plants having improved stress resistance to drought and/or freeze, possessing larger size attributes than wild-type counterpart varieties, and having increased tolerance to salt in the soil in which they are growing, and which provide for increased biomass of useful components.

#### **SUMMARY OF THE INVENTION**

The present invention discloses a transgenic plant having upregulated expression of vacuolar pyrophosphatase. It has been found that plants displaying such upregulated activity are generally larger than wild-type counterparts, demonstrate improved stress resistance to drought and/or freeze, have increased tolerance to salt in the media in which they are growing, and display higher meristematic activity (cell division) leading to greater biomass in certain plant parts as compared to wild type plants.

Any suitable exogenous nucleic acid molecule which alters expression of vacuolar pyrophosphatase in the plant can be used to transform the transgenic plants in accord with the present invention. The exogenous nucleic acid can comprise nucleic acid that encodes a vacuolar pyrophosphatase protein (an exogenous vacuolar

pyrophosphatase), such as AVP1, a functional portion thereof (peptide, polypeptide), or a homologue thereof, and/or nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced. By "exogenous nucleic acid" it is meant a nucleic acid from a source other than the plant cell into which it is introduced, or into a plant or plant part from which the transgenic part was produced. The exogenous nucleic acid used for transformation can be RNA or DNA, (e.g., cDNA, genomic DNA). In addition, the exogenous nucleic acid can be circular or linear, double-stranded or single-stranded molecules. Single-stranded nucleic acid can be the sense strand or the anti-sense strand. By a "functional portion" of a nucleic acid that encodes a vacuolar pyrophosphatase protein it is meant a portion of the nucleic acid that encodes a protein or polypeptide which retains a functional characteristic of a vacuolar pyrophosphatase protein. In a particular embodiment, the nucleic acid encodes AVP1, a functional portion or a homologue thereof. The AVP1 nucleic acid may be obtained, for example, from Arabidopsis or any other plant, or synthetically synthesized.

Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced includes regulatory sequences (e.g., inducible, constitutive) which function in plants and antisense nucleic acid. Examples of regulatory sequences include promoters, enhancers. The nucleic acid can also include, for example, polyadenylation site, reporter gene and/or intron sequences and the like whose presence may not be necessary for function or expression of the nucleic acid but can provide improved expression and/or function of the nucleic acid by affecting, for example, transcription and/or stability (e.g., of mRNA). Such elements can be included in the nucleic acid molecule to obtain optimal performance of the nucleic acid.

The nucleic acid for use in the present invention can be obtained from a variety of sources using known methods. For example, the nucleic acid encoding a vacuolar pyrophosphatase (e.g., AVP1) for use in the present invention can be derived from a natural source, such as tobacco, bacteria, tomato or corn. In one embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that corresponds to a wild type of the transgenic plant. In another embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that does not correspond to a wild type of the transgenic plant. Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into

which the exogenous nucleic acid is introduced (*e.g.*, regulatory sequence) can also be chemically synthesized, recombinantly produced and/or obtained from commercial sources.

A variety of methods for introducing the nucleic acid of the present invention into plants are known to those of skill in the art. For example, *Agrobacterium*-mediated plant transformation, particle bombardment, microparticle bombardment (*e.g.*, U.S. Patent No. 4,945,050; U.S. Patent No. 5,100,792) protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos can be used. The exogenous nucleic acid can be introduced into any suitable cell(s) of the plant, such a root cell(s), stem cell(s) and/or leaf cell(s) of the plant.

Any suitable plant, including angiosperms, monocots and dicots, and gymnosperms, and algae can be used to produce the transgenic plants, tissue cultures or cell cultures of the present invention. For example, tomato, corn, tobacco, rice, sorghum, cucumber, lettuce, turf grass, ornamental (*e.g.*, larger flowers, larger leaves) and legume plants can be transformed as described herein to produce the transgenic plants of the present invention. In addition, the transgenic plants of the present invention can be grown in any medium which supports plant growth such as soil or water (hydroponically).

A transgenic plant of the present invention is preferably tolerant to high salt concentrations in soil. By the term "salt" it is meant to include any salt, that is a compound formed when hydrogen of an acid is replaced by a metal or its equivalent, and includes, without limitation, salts comprising monovalent and divalent toxic cations, NaCl, KCl, CaCl<sub>2</sub>, MgCl, CdCl, ZnCl, and sulfide salts.

Salt tolerance may be introduced into a plant of the present invention by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that expression is upregulated. Any suitable vacuolar pyrophosphatase, several of which have been cloned, can be used in the compositions and methods of the present invention (*e.g.*, Sarasian, Z., *et al.*, *Proc. Natl. Acad. Sci., USA*, 89:1775-1779 (1992); Jenslerchl, *et al.*, *Molec. Biol.*, 29: 833-840 (1995); Kim, Y., *et al.*, *Plant Physiol.*, 106:375-382 (1994)). In a particular embodiment, the present invention relates to a transgenic plant which is tolerant to salt comprising an exogenous nucleic acid construct which is designed to overexpress AVP1 (Sarasian, Z., *et al.*, *Proc. Natl. Acad. Sci., USA*, 89:1775-1779 (1992)). Transformation of the plant cells



may be carried out in a whole plant, seeds, leaves, roots or any other plant part. Such transgenic plants are preferably altered such that they grow in a concentration of salt that inhibits growth of a corresponding non-transgenic plant. Transgenic progeny of the transgenic plants, seeds produced by the transgenic plant and progeny transgenic plants grown from the transgenic seed, which are also the subject of the present invention, advantageously carry such salt tolerant trait. Plants may be regenerated from transformed cells to yield transgenic plants, which may be screened for certain levels of salt tolerance. In a preferred embodiment, the exogenous nucleic acid encodes AVP1, or a homologue thereof. Preferably expression of the vacuolar pyrophosphatase in the plant is enhanced to an extent that the transgenic plant is tolerant to sodium chloride (NaCl) when the NaCl concentration is from about 0.2M to about 0.3M. A transgenic plant capable of growing in salt water may also be produced by introducing into one or more cells of a plant nucleic acid which upregulates expression of vacuolar pyrophosphatase in the plant to yield transformed cells. As used herein, "salt water" includes water characterized by the presence of salt, and preferably wherein the concentration of salt in the water is from about 0.2M to about 0.4M. In one embodiment, salt water refers to sea water.

The transgenic plants of the present invention can also be used to produce double transgenic plants which are tolerant to salt (about 0.2M to about 0.4M salt concentration). In one embodiment, the present invention relates to a double transgenic plant which is tolerant to salt comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of a vacuolar pyrophosphatase and an  $\text{Na}^+/\text{H}^+$  antiporter in the plant. The vacuolar pyrophosphatase in an advantageous construct is AVP1, or a homologue thereof, and the  $\text{Na}^+/\text{H}^+$  antiporter is AtNHX1, or a homologue thereof. The present invention also encompasses transgenic progeny of the double transgenic plant, as well as seeds produced by the transgenic plant and a progeny transgenic plant grown from the seed.

Drought and/or freeze tolerance may also be introduced into plants by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that such expression is upregulated. In a preferred embodiment there is provided a substantially drought and/or freeze resistant transgenic plant which comprises a genome having one or more exogenously introduced vacuolar  $\text{H}^+$ -translocating pump genes. A particularly preferred fertile transgenic plant

eliciting drought and/or freeze tolerance, as well as the ability to grow in saline soils, comprises an isolated exogenous chimeric DNA construct encoding vacuolar  $H^+$ -translocating pump, preferably operably linked to a promoter, such as the 35-S promoter or any other strong promoter, including, without limitation, tissue specific promoters. The  
5 transgenic plant may contain a polynucleotide sequence comprising an exogenous tonoplast pyrophosphate  $H^+$  pump gene operably linked to a promoter. In yet another particularly preferred drought and/or freeze resistant transgenic plant having the capacity to grow in saline soils, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphate  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S  
10 promoter. A particularly preferred tonoplast pyrophosphate  $H^+$  pump gene is the AVP1 gene.

Upregulation of expression of vacuolar pyrophosphatase by the methods described above may also be used to provide a plant having larger vegetative and/or sexual organs than wild type counterpart plants. That is, the present invention provides for a  
15 method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby  
20 producing a transgenic plant which is larger than its corresponding wild type plant. Also encompassed by the present invention is a method of making a transgenic plant (*e.g.*, an ornamental plant) having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells.

Upregulation of expression of vacuolar pyrophosphatase by the methods described above may also be used to provide a plant with meristematic activity cell division rate which is enhanced over wild-type counterpart plants. As would be  
25 recognized by one of ordinary skill in the art, meristems are central to higher plant development, as almost all post-embryonic organs, including roots, leaves, flowers and axillary meristems and cambium are initiated by either shoot or root meristems. Increased  
30 meristematic activity results in higher biomass in one or more aspects of plant structure, as evidenced by dry weight of plant structure, root structure as well as stem structure.

Increased meristemic activity is hypothesized to result in increased rate of shoot regeneration in root, leaf, hypocotyl, and cotyledon explants, and increased overall plant growth rate.

The present inventor has discovered that overexpression of a  
5 pyrophosphate driven proton ( $H^+$ ) pump at the vacuole leads to a greater proton pumping capacity that results in a greater ion uptake into the vacuoles that lowers the osmotic potential of the cells, and also leads to an increase in the capacity of plant cells to divide and multiply. As would be understood by one of ordinary skill in the art, such finding can have great commercial importance, e.g., reducing time for wood, corn etc. production by  
10 transforming cells so as to overexpress such pumps, and enhancing shoot regeneration capacity in plants with poor or slow regeneration capacity, such as woody plants, crops, e.g. corn, and ornamentals e.g., orchids. Overexpression so as to produce such enhanced cell division and multiplication may be performed using any of the construct described herein. While a number of inducible promoters and tissue specific promoters may be used  
15 to trigger overexpression of the gene and/or homologues of the gene, a preferred construct includes a tonoplast pyrophosphate driven  $H^+$  pump gene (*AVP-1*) operably linked to a chimeric promoter (e.g., double tandem enhancer of 35S promoter) designed to overexpress *AVP-1*.

There is also disclosed in the present invention novel gene cassettes  
20 including cassettes comprising a tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a chimeric promoter. A novel gene cassette comprising an exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a promoter, as well as novel coding sequences comprising an exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter. Preferably such coding  
25 sequence is designed to overexpress *AVP1*.

There is also disclosed in the present invention novel expression vectors including an expression vector containing a polynucleotide sequence comprising a  
exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter and further operatively linked to a multiple cloning  
30 site, and an expression vector containing a polynucleotide sequence comprising a exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double

tandem enhancer of the 35S promoter and further operatively linked to a heterologous coding sequence.

It is recognized by the present inventor, that the disclosed invention may have application to any plant, including, without limitation, crop plants, ornamental plants, grasses, shrubs, or any other plant found useful or pleasing to man, including having application to monocots, dicots, angiosperms, gymnosperms, and algae.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

The above description, as well as further objects, features and advantages present invention will be more fully understood with reference to the following detailed description when taken in conjunction with the accompanying drawings, wherein:

10 *Fig. 1A* is an overhead view of representative (out of 10 plants each) wild type (WT) and two independent transgenic lines (1' and 2') grown hydroponically for seven weeks on a 10 hour light/dark cycle;

15 *Figs. 1B(1), 1B(2) and 1B(3)* are a photomicrographs of the root and root hairs of representative five day old seedlings obtained from representative WT, 1' and 2' of *Fig. 1A* grown parallel to the surface on vertical plant nutrient agar plates;

*Fig. 1C* is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (1' and 2') overexpressing AVP-1;

20 *Fig. 2* is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') after exposure to 7 days of water deficit stress.

*Fig. 3* is a perspective view of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil.

25 *Fig. 4A* is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment; Nhx1 ( $\text{Na}^+/\text{H}^+$  antiporter), Vmal (vacuolar membrane  $\text{H}^+$ -ATPase), Gef1 (yeast CLC chloride channel), Ena1 (plasma membrane  $\text{Na}^+$ -ATPase).

30 *Fig. 4B* is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment shown in *Fig. 4A*, which also includes Avp1 (*A. thaliana* vacuolar pyrophosphate-energized proton pump).

*Fig. 5A and Fig. 5B* are bar graphs showing the intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type yeast strains and of yeast strains carrying various mutations affecting sodium tolerance wherein the values are the mean of two determinations, and the bars represent the standard deviations.

5        *Fig. 6* is an overhead view of shoot regeneration from root and cotyledon explants from wild-type (WT) and AVP-1 overexpressing (1' and 2') *Arabidopsis* plants, cultured in the SIM medium of *Fig. 13*.

*Fig. 7* is a bar graph of  $\text{Na}^+$  and  $\text{K}^+$  content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil.

10       *Fig. 8* is a graph of the uptake of calcium into the 35SAVP-1 transgenic vacuolar membrane vesicles (squares) of 2' of *Fig. 3* versus calcium uptake into vesicles obtained from wild type (WT) of *Fig. 3*.

*Figs. 9A and 9B* are illustrations demonstrating the 35SAVP-1 theorized mechanism for a higher accumulation of solids into vacuoles via a proton driven function versus that of WT vacuoles.

15       *Fig. 10* is an overhead view of the leave foliage of wild type versus transgenic (1' and 2') AVP-1 overexpressing *Arabidopsis* plants with leaves positioned with respect to one another according to age.

*Figs. 11A and 11B* are overhead views of watered (distilled water) leaves from wild type and transgenic (1' and 2') *Arabidopsis* plants overexpressing AVP-1 demonstrating growth of root structures.

*Fig. 12* is an overhead view of shoot regeneration in representative wild type petunia leave cuttings (WT) versus representative transgenic petunia plant leave cuttings overexpressing AVP-1 (35-S AVP-1).

25       *Fig. 13* is an overhead view of shoot regeneration from 5-day old cotyledons placed in SIM induction medium obtained from wild type (WT) and AVP-1 overexpressing (1' and 2') *Arabidiopsis* plants.

*Fig. 14* is bar graph of osmotic potential of fully hydrated leaves from WT and two AVP-1 overexpressing lines (1' and 2') from *Arabidopsis* plants.

30       *Fig. 15* is an overhead view of petunia explants incubated on MS medium showing callus induction from the explants after six weeks of incubation.

### DETAILED DESCRIPTION OF THE INVENTION

Since plant vacuoles constitute 40 to 99% of the total intracellular volume of a mature plant cell, changes in the size of the vacuole have dramatic effects upon cell size (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole* . (Academic Press Limited, 5 1997), vol. 25, pp. 298-337). The volume of the vacuole is controlled by ion and water fluxes mediated by pumps and transporters. In plants the driving force that triggers the movement of ions, solutes and water across membranes is a proton gradient. The activity of the vacuolar  $H^+$ -pumps results in luminal acidification and the establishment of a  $H^+$  electrochemical potential gradient across the vacuolar membrane, which powers the 10 secondary active transporters of inorganic ions, sugars, and organic acids. The activity of these transporters modulates cellular pH and ion homeostasis and leads to the accumulation of solutes required to generate the osmotic potential that promotes vacuolar expansion (H. Sze, X. Li, M. G. Palmgren, *The Plant Cell* 11, 677-689 (1999)).

There are three distinct pumps that generate proton electrochemical 15 gradients. One at the plasma membrane that extrudes  $H^+$  from the cell (PM  $H^+$ -ATPase) and two at the vacuolar membrane or other endomembrane compartments that acidify their lumen (the vacuolar type  $H^+$ -ATPase and  $H^+$ -PPase) (R. A. Leigh, in *The Plant Vacuole* L. a. Sanders, Ed. (Academic Press, San Diego, California, 1997), vol. 25, pp. 171-194.).

Previous work has shown that a decrease in the levels of the A subunit of 20 the vacuolar  $H^+$ -ATPase of carrot, using an antisense construct, resulted in a plant with reduced cell expansion and altered leaf morphology (J. P. Gogarten, et al., *The Plant Cell* 4, 851-864 (1992)). The present inventor has hypothesized that an increased supply of  $H^+$  into the vacuole could accelerate cell expansion. Recently, based on the theory that as the availability of protons in the vacuolar function of ion accumulation, it has been 25 hypothesized by the same inventor that accumulation of solids in the vacuoles might be useful to protect against draught and to provide for a more freeze resistant plants.

The present inventor has recognized that plants have a number of vacuolar  $H^+$ -translocating pumps, and that by upregulating their activity, increasing their expression, upregulating their transcription and/or translation, or increasing their copy 30 number that one can increase accumulation of solids in the vacuole due to an increase in the availability of protons in the vacuoles. The inventor tested this hypothesis by increasing the copy number of the vacuolar  $H^+$ -translocating pump, the inorganic

pyrophosphatase or V-PPase that consists of a single polypeptide (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole* . (Academic Press Limited, 1997), vol. 25, pp. 298-337). In *Arabidopsis* the V-PPase encoded by the *AVP-1* gene is capable of generating a  $H^+$  gradient across the vacuole membrane (tonoplast) similar in magnitude to that of the vacuolar  $H^+$ -ATPase (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* 89, 1775-1779 (1992)). As would be understood by one of ordinary skill in the art, similar genes in other plants should function in a similar manner.

It is known that  $H^+$ -PPase is the main proton pump of vacuolar membranes in growing tissue. The later may be due to the fact that in growing tissue, nucleic acids, DNA, RNAs, proteins and cellulose etc. are actively being synthesized for the construct of the new cells, and as a result, a large amount of PPi is produced as a by-product of these metabolic processes. The energy stored in the PPi molecule may be transformed into a different source of energy, namely a  $H^+$ -gradient across the vacuolar membrane. This  $H^+$ -gradient constitutes the driving force for the vacuolar accumulation of solutes that generate the sufficient osmotic differential that enables the plant cell to initiate growth. While the present invention is not limited in any manner to any particular hypothesis for the increased growth effects seen, the present inventor has hypothesized that in transgenic plants overexpressing AVP-1 that the greater number of  $H^+$ -PPases has a positive effect on the velocity of the generation of the  $H^+$  gradient, rendering a more active meristem.

In one embodiment, a construct comprising a vacuolar pyrophosphatase gene operably linked to a promoter designed to overexpress the vacuolar pyrophosphatase (e.g., an expression cassette) is used to produce the transgenic plants of the present invention. As used herein the term "overexpression" refers to greater expression/activity than occurs in the absence of the construct. In a particular embodiment, a construct comprising an *AVP1* gene operably linked to a chimeric promoter designed to overexpress *AVP1* is used to produce the transgenic plants of the present invention. More particularly, the present invention relates to a construct wherein the *AVP1* gene is operably linked to a double tandem enhancer of a 35S promoter.

The transgenic plants of the present invention may find utility other than those associated with the food value or ornamental value, industrial value such as, for example, wood production. For example, the transgenic plants of the present invention may uptake different or more ions than their wild-type counterparts. As discussed below,

studies with mutant yeast strains (*enal*) demonstrates that  $H^+$ -translocating pumps at the vacuole plays an important role in cation detoxification in higher plants (the plant components involved in an intracellular cation detoxification system being identified by complementing salt-sensitive mutants of the budding yeast *Saccharomyces cerevisiae*).

5 Transgenic plants and/or progeny thereof comprising exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant in accord with such studies may be used to bioremediate soil and growth medium. Such plants can be used to remove cations (*e.g.*, monovalent and/or divalent cations) from a medium which can support plant growth (*e.g.*, soil, water). For example, transformed plants of the present invention can be used to  
10 remove sodium (Na), lead (Pb), manganese (Mn) and/or calcium (Ca) ions from a medium which supports plant growth.

To demonstrate the effect that an increased supply of  $H^+$  into the vacuole would have on resistance to drought and/or freeze, and tolerance to salt growth, as well as size of the plants, the present inventor generated transgenic plants containing extra copies  
15 of a vacuolar proton pump, *AVP-1*.

*Arabidopsis thaliana* plants were transformed with constructs containing the *AVP-1* gene. Transgenic lines containing extra copies of this gene were then isolated. The *AVP-1*, open reading frame was cloned into the *Xma*I site of a modified pRT103 [R. Topfer, V. Matzeit, B. Gronenborn, J. Schell and H-H. Steinbiss, *Nucleic acid Research*  
20 15, 5890 (1987)]. This vector contains a tandem repeat of the 35-S promoter. A *Hind*III fragment containing the 35-S tandem promoter, AVP-1 ORF and the polyadenylation signal was subcloned into the *Hind*III site of the pPZP212 vector [ P. Hajdukiewicz, Z. Svab and P. Maliga, *Plant Molecular Biology* 25, 989-994 (1994)]. *Agrobacterium*-mediated transformation was performed via vacuum infiltration of flowering *Arabidopsis*  
25 *thaliana* (*ecotype columbia*). Transgenic plants were selected by plating seeds of the transformed plants on plant nutrient agar plates supplemented with 25 mg/liter kanamycin. Plants were subsequently selected for two generations to identify transgenic plant homozygous for the transgene.

*Fig. 1A* is an overhead picture of representative (out of 10 plants each) wild  
30 type (WT) and two independent transgenic lines (1' and 2') grown hydroponically for seven weeks on a 10 hour light/dark cycle. As can be seen in *Fig. 1A*, a visual comparison of transgenic line 2', which expresses the AVP-1 protein at highest level, transgenic line



1', and wild type (WT), demonstrates that the amount of AVP-1 correlates with the size of the plants. The mass of the transgenic plants was found to be greater than that of wild type. The dry weight of the entire transgenic plants, measured after 24 hours at 75°C (n = 4), for transgenic lines 1' and 2' was found to be 1.5 and 3 times greater than that of wild-type (WT).

*Fig. 1B(1), Fig. 1B(2) and Fig. 1B(3) are photomicrographs (magnification: times 40; bar length on photograph = 2mm) of the root and root hairs of representative five day old seedlings obtained from representative WT, 1' transgenic and 2' transgenic of Fig. 1A grown parallel to the surface on vertical plant nutrient agar plates. Seedlings of both transgenic lines 1' and 2' showed root hairs with an average length 40 and 70% larger than wild-type (WT) root hairs (Fig. 1B) (Root hair length along the whole root was determined from five members of each set of seedlings. An average of 80 root hairs per plant were measured). The length of the root hairs is correlated with the size of the vacuole, so the increased size of the root hair is likely to result from increased vacuolar volume. This compares with the *Arabidopsis* mutant *rdh3* which has been reported to have reduced vacuolar volume and is a short plant with abnormally short root hairs (M. E. Galway, J. W. J. Heckman, J. W. Schiefelbein, *Planta* 201, 209-218 (1997)). It is recognized that the increased root structure will have a positive impact on soil erosion, nitrogen fixation in legumes, and will aid in water and nutrient uptake by the plant.*

*Fig. 1C is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (1' and 2') overexpressing AVP-1. Total membrane fractions were isolated from shoots of eight week old wild type (WT) and AVP-1 transgenic plants (1' and 2') grown in a hydroponic media for 8 weeks. Homogenate of plant shoots were sequentially centrifuged for 15 and 30 min at 8,000 and 100,000 rpms respectively. The 100 mg membrane pellet was re-suspend in 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 1 mM PMSF Protein (10 ug) was separated on a 10% SDS-PAGE, electroblotted and immunostained with antibodies raised against a KLH-conjugated synthetic peptide corresponding to the putative hydrophilic loop IV of the AVP-1 protein (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* 89, 1775-1779 (1992)). PPase was detected by chemiluminescence. Fig. 1C illustrates that the transgenic lines (1' and 2') express AVP-1 protein at higher levels than the wild type (WT) (that is, 1' = 1.6 fold increase and 2' = 2.4 fold increase over WT).*

As wheat that has been deprived of water is rendered more drought tolerant by an increase in cell  $K^+$  content (from 100 mM to 300 mM) (S. Gupta, G. Berkowitz, P. Pier, *Plant Physiol* 89, 1358-1365 (1989)), it is hypothesized (but the invention is not hereby limited by such theory) that the increased drought resistance of the AVP-1 transgenic plants may be a consequence of their higher vacuolar concentration of potassium that results in a increased water retention capability. Laboratory tests appear to confirm this.

Fig. 2 is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') after exposure to 7 days of water deficit stress. Wild type and transgenic plants overexpressing *AVP-1* (Fig 3A) were tested for drought tolerance (24°C). After 7 days of water deficit stress wild type (WT) plants withered, whereas plants from both *35S AVP-1* transgenic lines (1' and 2') were turgid and alive. Furthermore, when the drought stressed plants were then watered, transgenic plants pursued normal growth, bolted and set seeds, whereas wild type plants died. The relative water content of leaves from wild type and *35S AVP-1* transgenic plants were determined along the water deficit stress, demonstrating increased water retention by the transgenic lines as compared to the WT plants.

Fig. 14 is bar graph of osmotic potential of fully hydrated leaves from wild-type (WT) and two AVP-1 overexpressing lines (1' and 2') from *Arabidopsis* plants. The decreased osmotic potential in leaves of transgenic plants measured at a constant water content is consistent with the contention that AVP-1 overexpression results in increased solute accumulation, and therefore enhancing water retention capability.

While not illustrated in the accompanying illustrations, similar results may be seen with respect to freeze challenge ( $< 0^{\circ}\text{C}$ ) over a 24 hour or more period for a number of plant species. While not limited to such hypothesis, transgenic plants overexpressing AVP-1 (1' and 2') are believed to provide enhanced protection from freeze as compared to wild type (WT) plants due to the higher amounts of cations in the vacuoles. Higher amounts of cations confer a greater osmotic pressure that leads to a greater water retention capability endowing the plants not only with the ability to withstand low soil water potentials, but also providing greater protection from freezing that leads to significant desiccation of the plants.

*Fig. 3* is a perspective view of wild type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil. Five wild-type plants (WT) and five of the two AVP-1 overexpressing transgenic lines (1' and 2') were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 50 mM. The illustrations in *Fig. 3* corresponds to representative plants at the tenth day in the presence of 300 mM NaCl. *Fig. 3* illustrates that the two AVP-1 plant types (1' and 2') were significantly hardier in salty soil as compared to wild-type plants. The fact that genetically engineered *Arabidopsis thaliana* plants that overexpress either *AVP1* (the pyrophosphate-energized vacuolar membrane proton pump, this work) or *AtNHX1* (the  $\text{Na}^+/\text{H}^+$  antiporter, (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999)) and this work) are capable of growing in the presence of high NaCl concentrations strongly supports the strategy described herein. A double transgenic plant would be expected to demonstrate a further enhanced salt-tolerant phenotype. These *Arabidopsis thaliana* transporters or their counterparts may perform similar function in important agricultural crops. The increased size of 35S *AVP1 Arabidopsis* transgenic plants also contribute to potential yield increases in genetically engineered crops.

#### A Working Model of Cation Homeostasis in Plant Organelles

While the present invention is not limited to any particular hypothesis, the present inventor have developed a working model for cation homeostasis in plant organelles which may explain the unexpected results discovered with respect to the transgenic plants disclosed herein.

In plants, most of the transport processes are energized by the primary translocation of protons.  $\text{H}^+$ -translocating pumps located at the plasma membrane and tonoplast translocated  $\text{H}^+$  from the cytosol to extracellular and vacuolar compartments, respectively (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). The plant tonoplast contains two  $\text{H}^+$ -translocating pumps; the V-ATPase and the inorganic pyrophosphatase or V-PPase. Their action results in luminal acidification and the establishment of a  $\text{H}^+$  electrochemical potential gradient across the tonoplast (Davies, J.M., *et al.*, The Bioenergetics of Vacuolar  $\text{H}^+$  Pumps. In: *Plant*

*Vacuole*, pp. 340-363, Leigh, R.A., Sanders, D. (eds.), Academic Press, San Diego (1997)). The vacuolar membrane is implicated in a broad spectrum of physiological processes that include cytosolic pH stasis, compartmentation of regulatory  $\text{Ca}^{2+}$ , sequestration of toxic ions such as  $\text{Na}^+$ , turgor regulation, and nutrient storage and retrieval. The vacuole constitute 40 to 99% of the total intracellular volume of a mature plant cell. The vacuolar proton pumping pyrophosphatase is a universal and abundant component of plant tonoplast capable of generating a steady-state trans-tonoplast  $\text{H}^+$  electrochemical potential similar or greater than the one generated by the V-ATPase (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). Pyrophosphate (PPi) is a by-product in the activation or polymerization steps of a wide range of biosynthetic pathways and in plants serves as an alternative energy donor to ATP for sucrose mobilization via sucrose synthetase, for glycolysis via PPi: fructose-6-phosphate phosphotransferase and for tonoplast energization via the vacuolar proton pumping pyrophosphatase (Stitt, M., *Bot. Acta* 111:167-175 (1998)).

Most of intracellular organelles, including clathrin-coated vesicles, endosomes, Golgi membranes and vacuoles have acidic interiors (Xie, X. S., *et al.*, *J. Biol. Chem.*, 264:18870-18873 (1989)). This acidification is mediated by a proton-translocating electrogenic ATPase and in plant vacuoles also via a pyrophosphate-driven proton pump V-PPase (Davies, J.M., *et al.*, The Bioenergetics of Vacuolar  $\text{H}^+$  Pumps. In: Leigh R.A., Sanders, D., (eds) *The Plant Vacuole*, pp. 340-363, Academic Press, San Diego (1997); Zhen, R.G., *et al.*, "The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane Academic Press Limited (1997)). There exists a requirement of anion transport to maintain net electroneutrality (al-Awqati, A., *Curr. Opin. Cell. Biol.*, 7:504-508 (1995)).

Fig. 4A is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment; Nhxl ( $\text{Na}^+/\text{H}^+$  antiporter), Vmal (vacuolar membrane  $\text{H}^+$ -ATPase), Gef1 (yeast CLC chloride channel), Ena1 (plasma membrane  $\text{Na}^+$ -ATPase). The yeast member of the CLC voltage-gated chloride channel superfamily, Gef1, is required for copper loading in late-Golgi vesicles and for cation sequestration in the pre-vacuolar compartment in yeast (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998); Gaxiola, R.A., *et*

al., *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999); Example 1). Furthermore, it has been shown that the defects of *gef1* mutants can be suppressed by the introduction of the prototype member of the CLC superfamily, the *Torpedo marmorata* CLC-0 or by the introduction of *Arabidopsis thaliana* CLC-c and CLC-d chloride channel genes (Hechenberger, M., et al., *J. Biol. Chem.*, 271:33632-33638 (1996); Gaxiola, R.A., et al.,  
5 *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)). Fig. 4B is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment shown in Figure 4A, which also includes Avp1 (*A. thaliana* vacuolar pyrophosphate-energized proton pump).

10 While not wishing to be bound by theory, two observations led to the proposal of the model for Na<sup>+</sup> sequestration in yeast illustrated in Figs. 4A and 4B. First, *gef1* mutants are sensitive to high NaCl concentrations. Second, the Na<sup>+</sup>/H<sup>+</sup> exchanger Nhx1 is localized to the pre-vacuolar compartment (Nass, R., et al., *J. Biol. Chem.*, 273:21054-21060 (1998)). The model proposed in Figs. 4A and 4B posits that Na<sup>+</sup>  
15 sequestration by Nhx1 depends on the vacuolar H<sup>+</sup>-ATPase and Gef1, the chloride channel. Gef1-mediated anion influx allows the establishment by the vacuolar H<sup>+</sup>-ATPase of a proton gradient sufficient in magnitude to drive the uphill accumulation of Na<sup>+</sup> via Na<sup>+</sup>/H<sup>+</sup> exchange.

Based on such models, it was theorized that increasing the influx of protons  
20 into the postulated endosomal compartment should improve Na<sup>+</sup> sequestration via the Nhx1 exchanger. In order to increase the H<sup>+</sup> availability the *A. thaliana* gain-of-function mutant gene *AVP1-D* that codes for the vacuolar pyrophosphate-energized proton pump was expressed (Figure 3B) (Zhen, R.G., et al., *J. Biol. Chem.*, 272:22340-22348 (1997)). This plant pump expressed in yeast restored the Na<sup>+</sup> resistance of the test strain, but only if  
25 the strain had functional *NHX1* and *GEF1* genes. Furthermore, Gef1p and Nhx1p co-localize within a common organelle, the pre-vacuolar compartment (Gaxiola, R.A., et al., *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). These results strongly support the model in Figs. 4A and 4B and indicate that the yeast pre-vacuolar compartment can be used to identify the elusive plant transporters involved in intracellular sodium detoxification.

30 The model set forth in Figs. 4A and 4B is entirely consistent with the physiological data on the role of the vacuole in cation detoxification in higher plants. Yeast and plant cells share pathways and signals for the trafficking of vesicles from the

Golgi network to the vacuole (Neuhaus, J.M., *et al.*, *Plant Mol. Biol.*, 38:127-144 (1998); (Paris, N., *et al.*, *Plant Physiol.*, 115:29-39 (1997); Sato, M.H., *et al.*, *J. Biol. Chem.*, 272:24530-24535 (1997); Vitale, A.V., *et al.*, *Trends Plant Sci.*, 4:148-154 (1999)). Studies were therefore undertaken in yeast to identify the role of the vacuole in cation  
5 detoxification in higher plants.

#### **Studies of Cation Sequestration Mechanism in Yeast**

##### **Example 1: Functionality of AtNhxl and Avp1 in Yeast Strains**

To test the sequestration models set forth in *Figs. 4A and 4B*, mutant yeast strains (*enal*) lacking the plasma membrane sodium efflux pump, which therefore must  
10 rely on the internal detoxification system in order to grow on high salt, were constructed. The sequestration model (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998) and, Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)) of *Figs. 4A and 4B* predicts that the *enal* strain would become salt tolerant if one could enhance the availability of protons in the postulated endosomal compartment, that is with increased  
15 influx of protons, cytoplasmic  $\text{Na}^+$  would be sequestered via the Nhxl exchanger.

The yeast vacuolar ATPase is a multisubunit protein, so it is difficult to increase its activity by overexpressing any one of its subunits. Instead the same effect was achieved by increasing the influx of protons by expressing the *A. thaliana AVP1* gene in yeast. This gene encodes a single polypeptide that, when expressed in yeast, is capable of  
20 pumping protons into the lumen of the vacuole (Kim, E.J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:6128-6132 (1994)). To ensure maximum activity of this proton pump, the E229D gain-of-function mutant of the *AVP1* gene (*AVP1-D*) that has enhanced  $\text{H}^+$  pumping capability was expressed (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). Intracellular sodium and potassium content was determined for mutant and wild-  
25 type cells after growth on SD-ura medium with high NaCl content.

## Materials and Methods

### Yeast strains and Plasmids

Strains isogenic to W303 (*ura3-1 can1-100 leu2-3, 112trp1-1 his3-11*,  
5 (Gaxiola, R.A., *et al.*, *EMBO J.*, 11:3157-3164 (1992)) were employed. Plasmids pRG52  
(*-gef1::HIS3*) (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998))  
and pRG197 (*-nhx1::HIS3*) were used to construct the deletions of *GEF1* and *NHX1*  
genes, yielding strains RGY85 and RGY296, respectively. The *enal::HIS3* mutant was  
obtained from Fink Lab collection (L5709).

### Method of Transformation

Transformation of yeast cells was performed by using the lithium acetate  
method (Gietz, D., *et al.*, *Nucleic Acids Res.*, 20:1425 (1992)). Double mutants RGY324  
(*gef1::HIS3 enal::HIS3*), RGY326 (*nhx1::HIS3 enal::HIS3*), and RGY343 (*gef1::HIS3*  
*nhx1::HIS3*) were obtained by crossing the single-mutant strains. Double mutants were  
15 identified among the meiotic progeny by scoring for the phenotypes associated with each  
of the single mutants. Sporulation, tetrad dissection, and mating types were scored as  
described (Guthrie C. and Fink, G.R., *Guide to Yeast Genetics and Molecular Biology*  
(Academic, San Diego (1991)). Cells were grown in YPD (1% yeast/2%peptone/2%  
dextrose; Difco), YPGAL (1% yeast/2%peptone/2% galactose; Difco), SD (Difco;  
20 Synthetic medium with 2% Dextrose), or APG (APG is a synthetic minimal medium  
containing 10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM MgSO<sub>4</sub>, 1 mM  
KCl, 0.2 mM CaCl<sub>2</sub>, and trace minerals and vitamins) (Rodriguez-Navarro, A. and Ramos,  
J., *J. Bacteriol.*, 159:940-945 (1984)). MnCl<sub>2</sub> (Sigma), tetramethylammonium chloride  
(Sigma), NaCl (Sigma), or hygromycin-B (Sigma) were added as indicated.

25 Wild type, L5709 (*enal::HIS3*), RGY324 (*gef1::HIS3 enal::HIS3*), and  
RGY326 (*nhx1::HIS3 enal::HIS3*) strains were transformed with pYES2 vector  
(Invitrogen) and plasmid pYES2-*AVP1*-E229D described in Zhen, R.G., *et al.*, *J. Biol.*  
*Chem.*, 272:22340-22348 (1997). The strain RGY343 (*gef1::HIS3 nhx1::HIS3*), used for  
histochemical analysis, was transformed with pRG151 (*GEF1-GFP*) (Gaxiola, R.A., *et al.*  
30 *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)) and with pRIN73 [*NHX1-(HA)<sub>3</sub>*] (Nass,  
R., and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)).

Wild-type and RGY296 (*nhx1::HIS3*) strains were transformed with vector pAD4 (Ballester, R., *et al.*, *Cell*, 59:681-686 (1989)). RGY296 (*nhx1::HIS3*) was transformed with pRG308 (*ADH1::AtNHX1*) (see *Cloning of AtNHX1*).

#### Determination of Intracellular Sodium and Potassium Content

5 Cells were grown overnight in SD-ura medium (Difco; synthetic medium with 2% dextrose without uracil). YPGAL (1% yeast extract/2% peptone/2% galactose; Difco) media was inoculated with the overnight stocks and grow to an  $A_{600}$  of 0.6. At this OD, NaCl was added to a final concentration of 0.7 M. The cells were incubated for 6 h, harvested by centrifugation, washed two times with 1.1 M sorbitol and 20 mM  $MgCl_2$ , and  
10 entracted with water for 30 min at 95°C.

The amount of  $Na^+$  and  $K^+$  in cells was determined at the University of Georgia Chemical Analysis Laboratory by an Inductively Coupled Plasma-MS (see world wide web at [rserv/uga.edu/rsnew/chemicalanalysis/](http://rserv/uga.edu/rsnew/chemicalanalysis/)). Intracellular cation concentrations were estimated as described (Gaxiola, R.A., *et al.*, *EMBO J.*, 11:3157-3164 (1992)) by  
15 using the intracellular water value calculated for cells grown in 1M NaCl.

#### Immunofluorescence

The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in SD-ura, -leu medium (Difco; synthetic medium with 2% dextrose without uracil and leucin) to mid-logarithmic phase, 0.1 mg/ml hygromycin B was added, and the culture was incubated for  
20 1 h at 30°C. Cells were fixed with 3.7% formaldehyde (Sigma) for 45 min at room temperature without agitation. Spheroplast formation, permeablization, washing, and antibody incubation was performed as described (Pringle, J., *et al.*, in *Immunofluorescence Methods for Yeast*, eds. Guthrie, C. And Fink, G.F. (Academic, Sand Diego), Vol. 194 pp.565-602 (1991)). MAB HA11 used as first antibody was from Babco  
25 (Richmond, CA). Cy3-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch. 4',6-Diamidino-2-phenylindole (Sigma) was added to mounting medium to stain mitochondrial and nuclear DNA.

#### Subcellular Fractionation and Western Analysis

The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in APG medium  
30 (pH 7.0), and lysates fractionated on a 10-step sucrose density gradient as described (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Aliquots of individual fractions (100  $\mu$ g) were subjected to SDS/PAGE and transferred to nitrocellulose as described



(Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Western blots were probed with monoclonal anti-GFP (green fluorescent protein) antibody (1:10,000 dilution; CLONTECH), anti-hemagglutinin antibody (1:10,000 dilution: Boehringer Mannheim), and peroxidase-coupled goat anti-mouse antibody (1:5,000;) and developed by using the  
5 ECL enhanced chemiluminescence system (Amersham Pharmacia).

#### Cloning of *AtNHX1*

*AtNHX1* was cloned from a phage cDNA library of *A. thaliana* (Kieber, J.J., *et al.*, *Cell*, 72:427-441 (1993)) (obtained from the Arabidopsis Biological Resource Center) by probing with an expressed sequence tag (Arabidopsis Biological Resources  
10 Center, DNA Stock Center) containing a partial clone. A full-length clone (2.1 kB) was ligated into vector pSK2 (Stratagene) at the *NotI* site, generating plasmid pRG293. The *AtNHX1* ORF was amplified via PCR by using pRG293 as template and GGCCCGGGATGGATTCTCTAGTGTCTGAACTGCCTTCG (SEQ ID NO: 5) (italicized bases correspond to nucleotides 1-30 of the ORF) and T7 oligonucleotides. The PCR  
15 product was then digested with *XbaI* and *SalI* and ligated into pAD4 vector generating plasmid pRG308. The *AtNHX1* ORF was sequenced to verify the fidelity of the PCR product. The full-length sequence is longer than the ORF reported by the Arabidopsis Genome Initiative (A TM021B04.4), and has been deposited in GenBank (accession no. AF106324).

#### Cloning of AVP1-D

Vector pYES2 (Invitrogen) was introduced into wild-type, *enal*, *enal nhx1*, and *enal gef1* mutants. Plasmid pYes2-AVP1-D (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)) was introduced into *enal*, *enal nhx1*, and *enal gef1* mutants. Five-fold serial dilutions (starting at  $10^5$  cells) of each strain were plated on YPGAL (1%  
25 yeast extract/2% peptone/2% galactose) with or without 0.5 M NaCl and incubated at 30°C for 2 days. Exponentially growing cells (wild-type and *enal* transformed with pYES2 vector and *enal*, *enal nhx1*, and *enal gef1* mutants carrying pYes2-AVP1-D) were exposed to 0.7M NaCl for 6 hours. Total cell extracts were prepared, and  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined.

#### Results

30 The *enal* mutant of the above construct lacks the plasma membrane sodium efflux pump and therefore must rely on the internal detoxification system to overcome

sodium toxicity. Growth of the *enal* strain is sensitive to low concentrations of sodium (200 mM), concentrations that do not inhibit the growth of wild-type strains. Overexpression of *AVP1-D* restored salt tolerance to salt-sensitive *enal* mutants. The restoration of salt tolerance to an *enal* strain by *AVP1-D* requires functional *NHX1* and *GEF1* genes: *enalnhx1 AVP1-D* and *enal gef1 AVP1-D* strains are salt sensitive.

*Fig. 5A* and *Fig. 5B* are bar graphs showing the intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type yeast strains and of yeast strains carrying various mutations affecting sodium tolerance wherein the values are the mean of two determinations, and the bars represent the standard deviations. The intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type strains and of strains carrying various mutations affecting sodium tolerance were determined after 6 h of exposure to media supplemented with 0.7 M NaCl. The intracellular  $\text{Na}^+$  content in the *enal* mutant was seen to be 8-fold higher than in the wild-type strain. There was seen to be a consistent reduction in total cell  $\text{Na}^+$  in the *enal AVP-D* strain. The reason for this reduction is unknown. The *enal AVP-D* strain was found to be salt-resistant, even though its intracellular  $\text{Na}^+$  content was 4-fold higher than that of the wild type. In *enal AVP1-D* strains lacking either *gef1* or *nhx1* (i.e., *enal gef1* or *enal nhx1*), the  $\text{Na}^+$  content was not reduced to the extent that it was in the *GEF1 NHX1* strain. Taken together, the genetic and physiological data are consistent with the model that *Nhx1*, *Gef1* and *Avp1* cooperate to sequester sodium internally. As can be seen in the graphs, the *Arabidopsis* Vacuolar  $\text{H}^+$ -Pyrophosphatase (*Avp1*) was evidenced to confer salt tolerance to yeast *enal* mutants.

The intracellular  $\text{K}^+$  content was found to correlate with salt tolerance and is inversely correlated with the  $\text{Na}^+$  content of the strains (*Fig. 4B*). The wild-type  $\text{K}^+$  concentration was at 100 mM, but was reduced to 20 mM in the *enal* mutant. Interestingly, in an *enal* strain that overexpresses the *AVP1-D* gene, the intracellular concentration of  $\text{K}^+$  was restored almost to wild-type levels (*Fig. 4B*). However, *AVP1-D* overexpression failed to restore wild-type levels of intracellular potassium unless both *NHX1* and *GEF1* were functional (See, the double mutants *enal nhx1* or *enal gef1* in *Fig. 4B*).

As shown herein, intracellular  $\text{Na}^+$  detoxification in yeast requires functional  $\text{Na}^+/\text{H}^+$  exchanger (*Nhx1*) and chloride channel (*Gef1*), and they co-localize to a pre-vacuolar compartment (Gaxiola, R.A., et al., *Proc. Natl. Acad. Sci. USA*, 96:1480-

1485 (1999)). When the *Arabidopsis thaliana* homologue of the yeast *NHX1* gene (*AtNHX1*) was cloned and its function in the *nhx 1* yeast mutant tested, the *AtNHX1* gene was found to be able to suppress partially the cation sensitivity phenotypes of *nhx1* mutants. Fig. 6 is alignment of the deduced amino acid sequences of NhX1 homologue from *Arabidopsis AtNHX1* (SEQ ID NO:1), human *HsNHE-6* (SEQ ID NO: 2) and yeast *ScNHX1* (SEQ ID NO:3); identical residues are in black boxes, and dashes indicate gaps in the sequence, \* above alignment denote putative amiloride binding site from human *NHE1* (<sup>163</sup>DVF-FLFLPPI<sup>173</sup>) (SEQ ID NO: 4).

Example 2: Functionality of , and Co-localization of, Gef1p and Nhx1p in Yeast Strains

10           The *NHX1* and *GEF1* genes, which have been identified as important in sodium detoxification, are also required for the detoxification of other cations. An investigation was made with respect to the viability of yeast strains mutant with respect to *gef1* and *nhx1* (*enal*) in the presence of toxic cations, in light of the model set forth in Figs. 4A and 4B.

15           The sequestration model postulates not only a functional connection between the anion channel Gef1 and sodium exchanger Nhx1 but also predicts that these two proteins co-localize within a common compartment. Because previous studies indicated that Nhx1 localizes to a pre-vacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)), experiments were also performed to determine  
20 whether Gef1 and Nhx1 proteins co-localize to this compartment.

Materials and Methods

          The strain RGY419 (*gef1 nhx1*) was transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-(HA)<sub>3</sub>. Transformants were grown in SD (Difco; synthetic medium with 2% dextrose). To determine the sensitivity of such transformants  
25 to toxic cations, five-fold serial dilutions (starting at 10<sup>5</sup> cells) of the indicated strains were grown at 30 °C for 2 days on YPD (1% yeast extract/2% peptone/2% dextrose) with the addition of either 3 mM MnCl<sub>2</sub>, 0.45 M tetramethylammonium (TMA), or 0.05 mg/ml hygromycin B (HYG) as indicated.

          Two studies were undertaken to demonstrate co-localization of *Gef1p* and  
30 *Nhx1p*.

          Distribution of fluorescence and immunodetection of subcellular fractions in *gef1 nhx1* cells transformed with two constructs: a *GEF1-GFP* fusion and a *NHX1-*

(HA)<sub>3</sub>-tagged fusion were determined. When the cells reached OD<sub>600</sub> = 0.5, hygromycin B (Sigma) was added to a final concentration of 0.1 mg/ml and the cells were incubated for 40 min at 30 °C. Cells were fixed and stained with antibodies to HA epitope and 4',6-diamidino-2-phenylindole (DAPI). Cells were viewed by charge-coupled device microscopy and optically sectioned by using a deconvolution algorithm (Scanalytics, Billerica, MA) (Kennedy, B.K., *et al.*, *Cell*, 89:381-391 (1997)); (Bar = 1 μm).

The migration properties of the *Gef1p* and *Nhx1p* in sucrose gradients was also determined to provide evidence of co-localization of Nhx1 (HA)<sub>3</sub> and GEF1-GFP. The strain RGY419 (*gef1 nhx1*) was transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-(HA)<sub>3</sub> and grown in APG medium (Rodriguez-Navarro, A. and Rea, P.A., *J. Biol. Chem.*, 159:940-945 (1984)). Such was converted to spheroplasts, lysed, and fractionated on a 10-step sucrose gradient (18-54%) as described (Sorin, A., *et al.*, *J. Biol. Chem.*, 272:9895-9901 (1997) and Antebi, A. and Fink, G.R., *Mol. Biol. Cell*, 3:633-654 (1992)). Western blots showed the distribution of Gef1-GFP and Nhx1-HA.

## 15        Results

*Gef1* mutants were found to be sensitive to 3 mM MnCl<sub>2</sub>, 0.45 M tetramethylammonium chloride and to 0.05 μg/ml hygromycin-B. The *nhx1* mutant was also found to be sensitive to tetramethylammonium chloride and hygromycin. The extreme sensitivity of the *nhx1* mutant to hygromycin may provide an important tool for assaying nhx1 function.

It was found that hemagglutinin (HA)-tagged Nhx1 and Gef1-GFP fusion protein co-localize as shown via epifluorescence deconvolution microscope. Persistence of signal coincidence on 90° rotation of the image further supports co-localization of the two transporter proteins in these cells. The co-localization of Nhx1 (HA)<sub>3</sub> and GEF1-GFP is also supported by the co-migration of the two proteins in sucrose density gradients of membrane preparations obtained from cells expressing the tagged proteins. The sedimentation behavior of the membrane fraction containing both proteins is consistent with that of a pre-vacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Gef1-GFP (but not Nhx1) is also present in Golgi fractions, consistent with previous studies (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998), Schwappach, B., *et al.*, *J. Biol. Chem.*, 273:15110-15118 (1998)).

Example 3: Capacity of *A. thaliana* Homolog of NHX1 to Suppress Hygromycin Sensitivity of Mutant Yeast

The yeast strain described herein provides an important tool for identifying genes that mediate salt tolerance in other organisms. To test the utility of this system, a sequence from *Arabidopsis* (See *Materials and Methods*) with very high homology to the *S. cerevisiae* NHX1 ORF was identified and used an expressed sequence tag (see *Materials and Methods*) to obtain a full-length clone of this *Arabidopsis* gene. An alignment of the amino acid sequences of Nhx1 homologues from *Arabidopsis* (AtNhx1), human (HsNhe6), and yeast (ScNhx1) reveals segments of amino acid identity and similarity within predicted transmembrane domains (Fig. 6A-C). However, it is important to note that despite these relationships, neither the C-terminal regions of AtNhx1 and ScNhx1 show a high degree of homology (Fig. 6A-C).

A characteristic of mammalian  $\text{Na}^+/\text{H}^+$  antiporters is their inhibition by amiloride. A putative amiloride binding site ( $^{163}\text{DVFFFLFLPPI}^{173}$ ) (SEQ ID NO: 4) has been defined via point mutants in the human NHE1 antiporter gene (Counillon, L., *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:4508-4512 (1993)). AtNhx1, HsNhe-6 and ScNhx1 have an almost identical sequence (Fig. 6). However, attempts to inhibit the activity of either Nhx1 or AtNhx1 in yeast cultures with amiloride were unsuccessful.

The extreme sensitivity of yeast *nhx1* mutants to hygromycin permitted the testing of whether the cloned *Arabidopsis* AtNHX1 ORF could provide  $\text{Na}^+/\text{H}^+$  exchange function in yeast. Vector pAD4 (Ballester, R., *et al.*, *Cell*, 59:681-686 (1989)) was introduced into wild-type and *nhx1* strains. Plasmid pRG308; ADH; AtNHX1 was introduced into *nhx1* mutants as indicated. Five-fold serial dilutions (starting at  $10^5$  cells) of the indicated strains were grown at 30°C for 2 days on YPD (-) or on YPD supplemented with 0.05 mg/ml hygromycin (+). Serial dilutions of the same strains were grown on APG medium (see *Materials and Methods*) (-) or on APG supplemented with 0.4 M NaCl (Rodriguez-Navarro, A. and Ramos, J., *J. Bacteriol.*, 159:940-945 (1984)).

The *At NHX1* gene is capable of suppressing the hygromycin sensitivity of the *nhx1* mutant. The *AtNHX1* gene also suppressed the NaCl sensitivity of *nhx1* mutant but only under conditions in which the  $\text{K}^+$  availability was reduced. However, *AtNHX1* was not capable of rescuing the  $\text{Na}^+$  sensitive growth phenotype of the double mutant *enal nhx1* overexpressing the *AVP1-D* gene.

Example 4: Generation of Gain-of-Function Yeast AtNHX Mutants

Materials and Methods

Gain of function mutants of the AtNHX that enhance salt tolerance may be generated in the *enal* yeast by mutagenizing the cloned gene to make a mutant library. This library may be used to transform the salt sensitive yeast mutant *enal* and clones with an enhanced salt tolerant phenotype.

Other genes that show similarity to the AtNHX1 gene, as reported by the Arabidopsis Genome Initiative (AGI), may also be expressed in the mutant yeast to form gain-of-function mutants. It is believed that some of these AtNHX1 homologues are plasma membrane transporters, so their function in yeast are frequently pH dependent, requiring precise composition and pH of the medium used for screening for success. Identification of plasma membrane transporters helps to engineer plants with an enhanced salt tolerance due to a reduced sodium uptake. In addition, plant cDNA expression libraries in yeast may be used to identify other families of transporters involved in NaCl detoxification.

To generate gain of function mutants of the AtNHX a method for introducing random mutations developed by Stratgene (Epicurian Coli XL1-Red competent Cells Cat#200129) may be used. The method involves the propagation of a cloned gene into a strain deficient in the three primary DNA repair pathways. The random mutation rate in this strain is about 5000-fold higher than that of wild-type. A library of the mutated AtNHX gene may be transformed into the *enal* yeast mutant and screened for salt tolerance. Yeast transformation was performed as described by Schiestl and coworkers (Gietz, D., *et al.*, *Nucl. Acid Res.* 20:1425 (1992), incorporated by reference in its entirety herein). An alternative to the XL1-Red random mutagenesis strategy is a PCR approach described by Fink and coworkers (Madhani, H.D., *et al.*, *Cell*, 91:673-684 (1997)).

To test AtNHX1 homologues the same strains and conditions may be as described in Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999). However, others may be employed. When dealing with plasma-membrane AtNHX1 homologues pH conditions of the assay media may be crucial.

### Results

The overexpression of the *A. Thaliana* gain-of-function mutant gene *AVP1-D* increases the intracellular detoxification capability in yeast (Gaxiola, R.A., *et al.*; *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). It is hypothesized (although the inventors are not limited by such hypothesis) that the latter is due to an increased influx of  $H^+$  into the vacuolar compartment thereby improving  $Na^+$  sequestration via the Nhx1 exchanger.

### Conclusions from Yeast Cation Sequestration Studies

The yeast studies described above provide evidence for the importance of the pre-vacuolar pH for intracellular  $Na^+$  sequestration in yeast. Overexpression of the plant  $H^+$ -pyrophosphatase (Avp1) confers salt tolerance to yeast only in those strains containing a functional chloride channel (Gef1) and the  $Na^+/H^+$  exchanger (Nhx1).

These data support a model in which the Nhx1  $Na^+/H^+$  exchanger acts in concert with the vacuolar ATPase and the GEF1 anion channel to sequester cations in a pre-vacuolar compartment. Several studies suggest that the pre-vacuolar compartment may be derived both from the plasma membrane and the late Golgi. These vesicles are likely involved in the assembly of the vacuole or delivery of cargo to this organelle. It is reasonable to expect that these pre-vacuolar vesicles detoxify cations by sequestration, thereby lowering their concentrations in the cytoplasm and in other organelles.

The yeast system described herein permits the functional assessment of diverse heterologous proteins in salt tolerance: chloride channels,  $H^+$  pumps, and  $Na^+/H^+$  exchangers and other cation/ $H^+$  exchangers or cation/bicarbonate symporters. The system is robust and flexible. The function of the *Arabidopsis* chloride channels (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998), Hechenberger, M., *et al.*, *J. Biol. Chem.*, 271:33632-33638 (1996)),  $H^+$  pump, and  $Na^+/H^+$  exchanger can be assayed in the corresponding yeast mutant. Despite the inability of *At NHX1* to suppress all the phenotypes of the yeast *nhx1* mutant, the fact that it suppresses some phenotypes, coupled with the DNA homology between *AtNHX1* and yeast *NHX1*, indicates that the plant gene carries out a similar function to that of the yeast homologue. The observation that the *AtNHX1* gene suppresses the sensitivity of the *nhx1* mutant to hygromycin but provides only a weak  $Na^+$  detoxification phenotype could be a consequence either of differential

regulation of the transporters in the two organisms or of distinct cation transport selectivities.

The regulation of *AtNHX1* by salt and the ability of the plant gene to suppress the yeast *nhx1* mutant suggest that the mechanism by which cations are detoxified in yeast and plants may be similar. Indeed, previous work suggested that vacuolar sodium accumulation in salt-tolerant plants may be mediated by a tonoplast  $\text{Na}^+/\text{H}^+$  antiporter that utilizes the proton-motive force generated by the vacuolar  $\text{H}^+$ -ATPase (V-ATPase) and/or  $\text{H}^+$ -translocating pyrophosphatase (V-Ppase; refs. Barkla, B.J., *et al.*, *Symp. Soc. Exp. Biol.*, 48:141-153 (1994), Zhen, R.G., *et al.*, *The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane* (Academic, San Diego), Kirsh, M., *et al.*, *Plant Mol. Biol.*, 32:543-547 (1996)).

The finding described herein that both *gef1* and *nhx1* mutants are hypersensitive to hygromycin indicate that the level of resistance to hygromycin depends on the function of the vacuolar and pre-vacuolar organelles. Yeast mutants impaired in  $\text{K}^+$  uptake (*trk1*) are hypersensitive to hygromycin (Madrid, R., *et al.*, *J. Biol. Chem.*, 273:14838-14844 (1998)); reduced  $\text{K}^+$  uptake hyperpolarizes the plasma membrane potential and drives the uptake of alkali cations such as hygromycin. Mutations that reduce the  $\text{H}^+$  pumping activity of the plasma membrane  $\text{H}^+$ -ATPase, Pmal, depolarize the plasma membrane potential and confer resistance to hygromycin (McCusker, J.H., *et al.*, *Mol. Cell. Biol.*, 7:4082-4088 (1987)). Thus, mutants such as *gef1* or *nhx1* that affect the pH or membrane potential of the vacuolar and pre-vacuolar compartments may be expected to affect hygromycin compartmentation.

#### Transgenic Plants With Upregulated Vacuolar $\text{H}^+$ -translocating Pump Activity

Further support for the role of the *Arabidopsis AtNHX1* gene in salt homeostasis is provided by the observation that its expression is induced in salt-stressed plants (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). *Arabidopsis thaliana*, in particular, has been used as a host model plant to demonstrate that overexpression of these genes results in salt tolerance in the plant. A recent report shows that the overexpression of *AtNHX1* gene in transgenic *Arabidopsis thaliana* promotes sustained growth in soil watered with 200 mM NaCl plus 1/8 M.S. salts under short-day cycle conditions (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999)). It is worth



noting that every addition of 1/8 M.S. salts provides 2.5 mM potassium reducing the stringency of the NaCl stress, and that a short-day cycle reduces oxidative stress.

Example 5: Enhanced Expression of AtNHX1 Gene in Salt-Stressed Plants

5 Materials and Methods

*A. thaliana* plants (ecotype Columbia) were grown aseptically on unsupplemented plant nutrient agar without sucrose (Haughn, G.W. and Somerville, C., *Mol. Gen. Genet.*, 204:430-434 (1986)) for 15 days at 19°C and under continuous illumination. NaCl or KCl was added to a final concentration of 250 mM, and the plants  
10 were incubated for 6 h. Total RNA from tissue of salt-treated and untreated plants was isolated (Niyogi, K.K. and Fink, G.R., *Plant Cell*, 4:721-733 (1992)), Hybond-N (Amersham) membranes were hybridized with a <sup>32</sup>P-Labeled DNA probe from plasmid pRG308. Hybridization was performed at 65°C overnight. Washes were performed at  
15 65°C with 0.2% standard saline citrate (SSC)/0.1% SDS (Ausebel, F., *et al.*, *Curr. Protocols in Mol. Biol.* (Wiley, NY) (1988)). 18S probe was used as loading control (Unfried, I., *et al.*, *Nucleic Acids Res.*, 17:7513 (1989)). MACBAS 2.4 program was used to quantify the relative amount of RNA.

Results

The NaCl stress increased *AtNHX1* mRNA levels 4.2-fold, whereas KCl  
20 promoted only a 2.8-fold increase. This increase in mRNA level produced by sodium resembles that described for the yeast *NHX1* gene (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). RNA tissue blot hybridized with *AtNHX1*. Ten micrograms of total RNA from 15-day old plants exposed to 250 mM NaCl or KCl for 6 h and a control grown without salt was subjected to electrophoresis on a denaturing formaldehyde gel.  
25 The blot was hybridized with a probe internal to *AtNHX1* ORF. An 18S ribosomal probe was used as a loading control.

Example 6: Salt tolerance of Transgenic plants overexpressing *AtNHX1*

Transgenic plants that overexpress the *AtNHX1* show sustained growth in  
30 soil watered with 200 mM sodium chloride. (Apsem M., *et al.*, *Science*, 285:1256-1258 (1999)).

Example 7: Salt tolerance of Transgenic plants overexpressing 35SAVP1

Materials and Methods

5 A transgenic *Arabidopsis thaliana* plant was engineered to overexpress the *AVP1* wild-type gene using the double tandem enhancer of the 35S promoter (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1987)). *AVP1* encodes the pyrophosphate-energized vacuolar membrane proton pump from *Arabidopsis* (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). Previous investigations suggest that the *AVP1* gene is present  
10 in a single copy in the genome of *Arabidopsis* (Kim, Y., *et al.*, *Plant Physiol.*, 106:375-382 (1994)), however, a sequence homologous, but not identical, to *AVP1* on chromosome one has been tentatively designated as ORF F9K20.2 on BAC F9K20 by the *Arabidopsis* Genome Initiative (AGI).

Transgenic plants that overexpress *AVP1* were generated using  
15 *Agrobacterium*-mediated plant transformation. The transgenic *AVP1* was expressed using a double tandem enhancer of the 35S promoter of CaMV (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1987)). 15 wild-type plants and 15 35SAVP1 transgenics were grown on a 24 hours-day cycle for 16 days. During this period plants were watered every 4 days with a diluted nutrient solution (1/8 M.S. salts). 200 mM NaCl was added to the watering  
20 solution at day 17 and at day 27 plants were watered with nutrient solution containing 250 mM NaCl. Plants were photographed 10 days after the last NaCl treatment. Identical conditions and treatment as described in Example 6 were used.

Results

Five different lines of 35SAVP1 plants showed an enhanced salt tolerance  
25 as compared to wild-type plants in the T2 stage. However, the most dramatic phenotype was apparent in the homozygous T3 plants. These transgenic plants are larger than wild-type plants. Furthermore, homozygous 35SAVP1 plants showed sustained growth in the presence of 250 mM NaCl plus 1/8 M.S. salts when grown in a 24 hours light regimen. When 35SAVP1 plants were grown under short-day cycle conditions (12 hour day/light  
30 cycle) sustained growth in the presence of 300 mM NaCl plus 1/8 M.S. salts was observed.

Example 8: Growth of Wild Type Plants and 35SAVP1 Transgenic Plants in Hydroponic Solution

The reduced availability of fresh water for standard agriculture may force the use of alternative agricultural arts. It is conceivable that with salt tolerant crops the use of hydroponics with seawater will create a new era in crop production. Hydroponic culture has been reported to increase plant growth and provide stress-free root and shoot material (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)). Another important advantage of hydroponic culture is that it allows one to alter the ionic composition in a more accurate manner than in soil. These advantages could be important for the physiological studies of salt stress.

Conditions for hydroponics culture of *Arabidopsis* plants were established and their performance in increasing concentrations of NaCl in their media were tested.

Materials and Methods

In one test, wild type and 35SAVP1 transgenic plants were hydroponically grown. Wild type and 35SAVP1 transgenic plants were grown in solution culture on a 12 hour light cycle for 65 days.

In another test, wild type and 35SAVP1 transgenic plants were grown in solution culture on a 12 hours light cycle for 20 days. Starting at day 21, NaCl concentration was increased in a stepwise fashion by 50 mM increments every 4 days. Plants were photographed after 4 days in the presence of 200 mM NaCl.

And yet in another hydroponic test, transgenic plants were challenged with a commercial seawater formula that contains the complete ionic composition present in the oceans. 35SAVP1, 35SAtnHX1 single and double transgenics were grown together with wildtype *Arabidopsis thaliana* plants under hydroponic conditions for four weeks in a short day illumination cycle (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)). Then every four days an equivalent to 50mM NaCl of Tropic Marin sea salt (worldwide web at thatpetplace.com) is added. This artificial sea water mix includes all of the other major and trace elements present in real sea water. Growth was monitored and physiological parameters, such as sodium content and distribution may be monitored.

Results

When wild type and 35SAVP1 transgenic plants were grown hydroponically the size differences in root, leaves and stems among wild type and

35SAVP1 transgenic plants were found to be dramatic, with 35SAVP1 transgenic plant parts being much larger.

When NaCl concentration were increased stepwise by 50 mM every 4 days (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999), 35SAVP1 transgenic plants appeared healthy in the presence of 200 mM NaCl while wild type controls showed severe deleterious effects in their leaves and stems.

35SAVP1, 35SAAtNHX1 single and double transgenics that were grown together with wildtype *Arabidopsis thaliana* plants under hydroponic conditions for four weeks in a short day illumination cycle (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)) and then challenged every four days with an equivalent to 50mM NaCl of Tropic Marin sea salt were found to grow in the sea salt solution better than wildtype *Arabidopsis thaliana* plants.

Example 9: Effect of Overexpression of Arabidopsis thaliana Proton Transporters in Tomato Plants

The effects of the overexpression of these *Arabidopsis thaliana* proton transporters (AVP1 and AtNHX1) in a more agriculturally important plant, the tomato plant, may be examined. It is believed that increasing the salt-tolerance of tomato plants will likely have important economic repercussions.

The tomato homologues of AVP1 and AtNHX1 may be isolated and the corresponding chimeras to overexpress them may be constructed (Bidone, S., *et al.*, *Eur. J. Biochem.*, 253: 20-26 (1998); Burbidge, A., *et al.*, *J. Exper. Botany*, 48:2111-2112. (1997)). The genes may be introduced via *Agrobacterium*-mediated infection of calli. Tissue culture methods may be used to regenerate transformed plants. The plants may be assayed for salt tolerance as well as physiological parameters, such as sodium content and distribution.

Tomato transformation with 35S AVP1 and with 35S AtNHX1 constructs may be performed as described by McCormick (McCormick, S., Transformation of tomato with *Agrobacterium tumefaciens*. In: *Plant Tissue Culture Manual*, pp. 1-9, Lindsey, K. (ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands (1991)). T0 and T1 transgenics may be analyzed by polymerase chain reaction and DNA gel blotting for the presence and copy number of AVP1 and AtNHX1 transgenes. Heterozygous and

homozygous plants may be identified after segregation analysis of each transgene within T1 seeds. Homozygous plants may be assayed for salt tolerance and as well as physiological parameters, such as sodium content and distribution. Degenerated oligos based on conserved sequences present in AVP1 and AtNHX1 homologues may be designed. These degenerated primers may be used in RT-PCR reactions with cDNAs made from poly(A)+RNA from tomato. The resulting PCR fragments may be used as probes to isolate the full length cDNA clones from commercial libraries (i.e. Stratagene Cat#936004). A similar strategy was described by Caboche and coworkers (Quesada, A., *et al.*, *Plant Mol. Biol.*, 34:265-274 (1997)).

10

### Results

Positive test results would indicate that the sequestration model described herein is also applicable to an important crop.

*Fig. 7* is a graph of  $\text{Na}^+$  and  $\text{K}^+$  content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil. Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (1' and 2') were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 50 mM. The photograph corresponds to plants at the tenth day in the presence of 300 mM NaCl. Parts of the plant above ground were harvested after 24 hours in the presence of 200 mM NaCl and their fresh weight measured. After 48 hours at 75°C, the dry weight was measured.  $\text{Na}^+$  and  $\text{K}^+$  content was determined by atomic absorption. Values in the graphs of *Fig. 4* are the mean  $\pm$  SE ( $n = 4$ ). As can be seen from the graphs  $\text{Na}^+$  and  $\text{K}^+$  content in the transgenic lines (1' and 2') was significantly higher than that of wild-type counterparts.

*Fig. 8* is a graph of the uptake of calcium into the 35SAVP-1 transgenic vacuolar membrane vesicles (squares) of 2' of *Fig. 4* versus calcium uptake into vesicles obtained from wild type (WT) of *Fig. 4*. Wild-type plants (open circles) and transgenic plants from line 2' of *Fig. 4* were grown hydroponically for nine weeks on a 10 hour light cycle. Vacuolar membrane vesicles were added to buffer containing 250 mM sorbitol, 25 mM BTP-Hepes pH 8.0, 50 mM KCl, 1.5 mM  $\text{MgSO}_4$  and 10  $\mu\text{M}$   $\text{Ca}^{++}$ . This mix was incubated at 20°C for 10 minutes before adding 200  $\mu\text{M}$  PPI to trigger the reaction.  $\text{Ca}^{++}$

ionophore A23187 was added to a final concentration of 5  $\mu\text{g/ml}$  to dissipate the  $\text{Ca}^{++}$  gradient. Aliquot (200  $\mu\text{l}$ ) were filtered at the indicated times and washed with cold buffer as described (11). As is evidenced by the graphs, the transgenic plants from line 2' have greater calcium uptake than wild-type plants.

5           The above data is consistent with the hypothesis that transgenic plants overexpressing *AVP-1* have an enhanced  $\text{H}^+$  pumping capability at their tonoplast and that an enhanced  $\text{H}^+$  supply results in greater ion accumulation in the vacuole through the action of  $\text{H}^+$ -driven ion transporters. To further support this theory,  $\text{Ca}^{++}$  uptake capability of wild type and transgenic vacuolar membrane vesicles was determined.

10           It is well documented that  $\text{Ca}^{++}$  enters the plant vacuole via a  $\text{Ca}^{++}/\text{H}^+$  antiporter (K. S. Schumaker, H. Sze, *Plant Physiol.* 79, 1111-1117 (1985)). Furthermore, the genes encoding the *Arabidopsis thaliana*  $\text{Ca}^{++}/\text{H}^+$  antiporters *CAX1* and *CAX2* have been isolated and characterized (K. D. Hirschi, R.-G. Zhen, K. W. Cunningham, P. A. Rea, G. R. Fink, *Proc. Natl. Acad. Sci. USA* 93, 8782-8786 (1996)). Fig. 8 shows that  
15  $\text{Ca}^{++}$  uptake in the *35SAVP-1* transgenic vacuolar membrane vesicles is 36% higher than it is in vesicles obtained from wild type. Application of the  $\text{Ca}^{++}$  ionophore A23 lowered the  $^{45}\text{Ca}^{++}$  counts to background levels demonstrating the tightness of the vesicles (Fig 8) (K. S. Schumaker, H. Sze, *Plant Physiol.* 79, 1111-1117 (1985)).

20           While not limited by such theory, a model consistent with the enhanced drought and freeze tolerance of the transgenic plants overexpressing the *AVP-1* gene is depicted in *Figs. 9A and 9B*. The model depicts how an increase in the number of *AVP-1* pumps in the vacuole of transgenic plants can provide more  $\text{H}^+$  that will permit the secondary transporters to import greater amounts of cations into the lumen of the vacuoles. Higher amounts of cations confer a greater osmotic pressure (See, *Fig. 14*) that  
25 leads to a greater water retention capability endowing plants to withstand low soil water potentials.

Example 10: Double Transgenic Plant with 35S AVP1 and 35S AtNHX1

Overexpression of the pyrophosphate-energized vacuolar membrane proton pump AVP1 likely increases the availability of  $H^+$  in the lumen of the vacuole, and the  
5 AtNHX1  $Na^+/H^+$  antiporter uses these  $H^+$  to sequester  $Na^+$  cations into the vacuole. Therefore, higher expression of these transporters likely maximizes the sequestration capability of the vacuole.

To generate transgenic *Arabidopsis* plants that overexpress both genes AVP1 and AtNHX1, T3 35S AVP1 plants may be used as females and T3 35S AtNHX1  
10 plants may be used as males. Female plants may be hand-emasculated and anthers from freshly opened flowers of donor plants are harvested. With these anthers the emasculated plants may be pollinated by touching the anthers onto the stigmas. The pollinated flowers may be labeled and any remaining opened or unopened flowers from the same female plant removed to avoid any confusion at harvest. The harvested seeds should be sterilized  
15 using a 50% sodium hypochloride solution and mixed vigorously for 5 minutes and rinsed with water thoroughly. The sterilized seeds may be stored in soft agar over night at 4°C. Then they may be sprinkled onto solidified kanamycin-hygromycin selective medium. The 35S AVP1 construct has the neomycin phosphotransferase II gene that confers kanamycin tolerance in plants while the 35S AtNHX1 construct has a modified  
20 hygromycin B phosphotransferase that confers hygromycin tolerance in plants. The resistant seedlings may be transplanted into soil and to the hydroponic media to be tested for their salt-tolerant phenotype.

A transgenic *Arabidopsis thaliana* plant to overexpress the *A. thaliana* gain-of-function mutant gene AVP1-D (Zhen, *et al.*, *J. Biol. Chem.*, 272:22340-22348  
25 (1997)) may be engineered using the same double tandem enhancer of the 35A promoter described above (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1997)). Plants overexpressing the gain of function mutant gene will likely show an enhanced phenotype. These plants may be characterized in parallel with the 35SAVP1, 35S AtNHX singles and doubles transgenics. The *A. thaliana* gain-of-function mutant gene AVP1-D may be  
30 subcloned into plasmid pRT103 carrying the 35S promoter and the polyadenylation signal of CaMV (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1997)). A HindIII fragment containing the chimeric 35SAVP-D gene may be subcloned into pBIBhyg (Becker, D.,

*Nucl. Acid Res.*, 18:203 (1990)). The resulting T-DNA vector may be transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and may be used for subsequent vacuum infiltration of *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al.*, *C.R. Jeances Acad. Sci. Ser. III Sci. Vie*, 316:1194-1199 (1993)). Integration may be confirmed on Southern blots of T3 plants and expression monitored on Northern blots of positive T3 plants.

Example 11: Comparative Transport Study With Vacuoles From The Roots Of Wild-Type and 35S AVP1 Transgenic Plants

A study may be undertaken to determine if the vacuoles of 35S AVP1 transgenic plants show a higher proton transport activity dependent on pyrophosphate. These determinations may be done with root and shoot tissues separately from plants grown hydroponically. The transgene could show a tissue-specific regulation despite the 35S promoter.

In order to compare the PPI-dependent  $H^+$  translocation activities of wild-type and 35S AVP1 transgenic plants sealed tonoplast-enriched vesicles from roots and leaves of the above plants may be prepared. The homogenization and differential centrifugation procedure described by Rea and Turner (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In: *Meth. Plant Biochem.*, pp. 385-405, Academic Press limited, London (1990)) may be followed.  $H^+$  translocation may be assayed fluorimetrically using acridine orange (2.5  $\mu$ M) as transmembrane pH difference indicator in assay media containing vacuole membrane-enriched vesicles as described by Rea and coworkers (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). The assay media contains 300- $\mu$ M Tris-PPI, 50mM KCl, 2.5  $\mu$ M acridine orange, 5mM Tris-Mes (pH 8.0). Intravesicular acidification may be triggered with the addition of 1.3 mM  $MgSO_4$  and terminated with the addition of the protonophore FCCP at 2.5  $\mu$ M. Fluorescence may be measured at excitation emission wavelengths of 495 and 540 nm, respectively, at a slit width of 5 nm (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 269:23342-23350 (1994)). A further test to support that the  $H^+$  translocation is AVP1 driven may be the addition of the specific inhibitor aminomethylenediphosphonate (Zhen, R.G., *et al.*, *Plant Physiol.*, 104:153-159 (1994)).



Example 12: Determination Of The  $\text{Na}^+/\text{K}^+$  Ratios In Leaves And Stems Of The  
Transgenic Plants

5

Toxic concentrations of NaCl build up first in the fully expanded leaves where NaCl is compartmentalized in the vacuoles. Exposure to NaCl can disrupt or reduce  $\text{K}^+$  uptake leading to  $\text{K}^+$  deficiency and growth inhibition (Wu, S.J., *et al.*, *Plant Cell*, 8:617-627 (1996). A cytosolic consequence of reduced  $\text{K}^+$  content and high  $\text{Na}^+$  is the inhibition of important enzymes. An example of such enzymes is the 3'(2'), 5'-bisphosphate nucleotidase of yeast whose activity is more sensitive to  $\text{Na}^+$  when  $\text{K}^+$  content is low (Murguia, J.R., *et al.*, *Science*, 267:232-234 (1995).

Measurements may be taken to demonstrate that the transgenic plants described herein have an increased vacuolar capacity to sequester  $\text{Na}^+$  in their leaves cells or elsewhere. To determine the  $\text{Na}^+/\text{K}^+$  ratios in leaves and stem S wild-type and 35S *AVP1* /35S *AtNHX1* double and single transgenics in hydroponic conditions (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997) may be grown. NaCl may be added to the growth media in a stepwise fashion starting with 50 mM up to 250mM (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999). At every point the rosette and the stems of the treated plants may be collected and their weight determined. The samples should be dried out in an oven at 80°C and their dry weight determined. The dry samples may be boiled in a determined volume of water and their  $\text{Na}^+$  and  $\text{K}^+$  contents determined via atomic absorption spectrophotometry (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999); Gaxiola, R., *et al.*, *Embo J.*, 11:3157-2164 (1992)).

25

Example 13: Determination Of Whether 35S *AVP1* Transgenic Plants Are Larger Because Their Cells Are Larger Or Because They Have More Cells,  
Or Both

30

The shoot meristems labeling index may be compared with one of the wild-type plants. Morphological and anatomical observations measuring and counting cells of leaves, roots and stems may be performed. To determine if 35S *AVP1* transgenic plants are larger because they have more cells, their shoot meristems labeling index may be compared with the one of wild-type plants.

To measure the DNA synthesis or cell proliferation 5-Bromo-2'-deoxy-uridine (BrdU) that can be incorporated into DNA in place of thymidine may be used. Cells that have incorporated BrdU into DNA may be detected using a monoclonal antibody against BrdU monoclonal antibody and an anti-mouse Ig-alkaline phosphatase as a second antibody. The bound anti-BrdU monoclonal antibody may be visualized by light microscopy and the ratio between DAPI stained and BrdU positives established. The protocol is a modification of the one published by Chiatante and coworkers (Levi, M., *et al.*, *Physiol. Plant.* 71:68-72 (1987)) and the BrdU labeling and detection kit II from Boehringer Mannheim. The plants may be exposed for different times to the BrdU labeling medium and then fixation, paraffin embedding and sectioning may be performed as described by Meyerowitz and coworkers (Drews, G., *et al.*, *Plant Mol. Biol. Rep.*, 5:242-250 (1988)).

For observation of leaf tissue, fresh tissues may be embedded in 5% agarose and slice them with a microslicer. For primary root observation, seedlings may be fixed for 4hr in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde at room temperature, dehydrated in graded ethanol series, permeate them with xylene, and infiltrate them with paraffin. Eight-micrometer sections may be stained with 0.05% toluidine blue and cells may be counted under a microscope. As an alternative for the visualization and determination of cell size the method described by Greenberg and coworkers (Rate, *et al.*, *The Plant Cell*, 11:1695-1708 (1999)) may be followed.

### Results

Microscopic studies indicate that the cells of the transgenic *Arabidopsis* plants are not larger, but that the number of cells is greater in the transgenics versus wild-type. Macroscopically it was seen that in the AVP-1 line-2' an average of eight more leaves in the rosette was noted over wild-type. F1 plants originated by crossing transgenic lines 1' and 2' displayed rosettes with larger leaves and increased amount of leaves than wild-type plants. *Fig. 10* depicts the foliage of wild type and transgenic (1' and 2') *Arabidopsis* plants overexpressing AVP-1 grown at 20°C under all white fluorescent light in 16 hours light/8 hours dark period cycle. Leaves depicted were carefully sectored with a scalpel when plants initiated to bolt and then ordered by size for comparison purposes. While having larger and more leaves than the wild-type plants, the transgenics were not

seen to have larger cell sizes. Such data is consistent with the hypothesis that the meristem is more active in transgenic AVP-1 overexpressers.

Dry weight of the transgenic *Arabidopsis* plants grown hydroponically, as compared to similarly grown wild-type plants, further indicates that cell mass increases irrespective any increased water uptake by the plant. An increase in dry mass weight was seen in both the root, rosette and stem structures as indicated in Table 1 below where values represent the mean values of six plants.

Table 1  
Dry Weight of *Arabidopsis* Plant Parts Grown Hydroponically

<u>PLANT PART</u>	<u>WILD-TYPE (WT)</u>	<u>1' TRANSGENIC</u>	<u>2' TRANSGENIC</u>
Roots	0.03 g	0.05 g	0.14 g
Rosette	0.10 g	0.20 g	0.60 g
Stems	0.40 g	0.50 g	0.80 g

**Example 14: Effect of Overexpression of AVP-1 on Hormonal Activity**

Increased meristematic activity and/or shoot organogenesis may be produced by increasing hormone availability together with AVP-1 overexpression.

The effect of overexpression of AVP-1 on hormone activity was adjudged placing true leaves from wild type (WT) and transgenic (1', 2') *Arabidopsis* plants overexpressing AVP-1 by careful sectoring with a scalpel. Leaves were placed on 5 layers of filter paper saturated with distilled water in Petri dishes. The Petri dishes were incubated at 20°C under cool white fluorescent light in a 16 h light/8 h dark cycles. As seen in *Figs. 11A* (view of leaves on the petri dishes) and *11B* (view of leaves from petri dishes of *Fig. 11A* positioned so as to more clearly set depict root structure developing from the leaves), leaves from transgenic plants remain greener and had an enhanced rooting capability. An enhanced rooting capability suggests increased auxin content and delayed senescence consistent with increased cytokinin levels.

**Example 15. Effect of Overexpression of AVP-1 on Callus Induction in Petunia Explants**  
Method

Petunia explants were incubated on MS medium which consists of MS salts (Gibco BRL), 1 mg/L nicotinic acid, 1 mg/L pyridoxin HCl, 1mg/L thiamine, 100 mg/L

myo-inositol, 3% sucrose, 1 mg/L 2,4-D(2,4-dichlorophenoxyacetic acid) and 0.5 mg/L 6-BA (6-benzylaminopurine). The medium was solidified with 0.7% agar and was adjusted to pH 5.8 before autoclave. The culture was incubated at 25°C in the dark in a growth chamber.

5

### Results

Transgenic petunia explants (35-S AVP-1) demonstrated significantly enhanced callus induction at 6 weeks of incubation as demonstrated in *Fig. 15*.

10

### Example 16: Effect of Overexpression of AVP-1 on Shoot Regeneration From Leave Segments

Leave segments grown in appropriate medium are known to be capable of generating shoot growth. A study was undertaken to determine the effect of overexpression of 35-S *AVP-1* on shoot regeneration in *Petunia* leaves.

15

Segments of leaves from regenerated transformed (35-S *AVP-1*) and control *Petunia* were used as explants for shoot regeneration. The leaves were cut with a sharp surgical blade into about 1 cm wide pieces. The explants were cultured in MS medium which included MS salts (Gibco), B5 vitamins (1 mg/L nicotinic acid, 1 mg/L pyridoxin HCl, 1 mg/L-thiamine and 100 mg/L myo-inositol), 3% sucrose, 2 mg/L 6-benzylaminopurine and 0.01 mg/L naphthaleneacetic acid, 0.7% agar, pH 5.8. The cultured segments were incubated at 25°C under cool white fluorescent light in a 16 h light/8 h dark period cycle.

25

As shown in *Fig. 12*, petunia leaves transformed with the *AVP-1* gene (35-S AVP-1 petri dish) under a constitutive promoter show an enhanced shoot regeneration capability over wild-type petunia leave segment (WT) similar to results with transgenic *Arabidopsis* plants. These results are consistent with the idea that overexpression of this vacuolar proton pump will improve the shoot regeneration capacity of any plant.

30

A blast search using *AVP-1* ORF as a probe showed this gene is highly conserved (rice = 86% identities; tobacco = 89% identities; barley = 86% identities; *Vitis vinifera* (grapes) = 82% identities, *Hordeum vulgare* (barley) = 86% identities and *Zea mays* (corn) = 90% identities). The latter indicates that overexpression of the AVP-1 gene would similarly cause such effects in other plant types.

Example 17: Effect of Overexpression of AVP-1 on Shoot and Root Regeneration From *Arabidopsis* Cotyledons

With appropriate medium, it is known that shoots and roots may be regenerated from cotyledons. Shoot and root regeneration from cotyledons was adjudged for wild-type (WT) and AVP-1 transgenic *Arabidopsis* (1' and 2').

Five (5) day old cotyledons were used as explants for regeneration. Explants were incubated on shoot induction medium (SIM) at 20°C under 16 h light/8 h dark period. The SIM contained MS salts (Gibco), B5 vitamins including 1 mg/L nicotinic acid, 1 mg/L pyridoxin HCl, 1 mg l-thiamine, and 100 mg/L myoinositol, 3% sucrose, 1 mg/L 6-(gamma-gama-dimethylallylamino)purine riboside (2-iP) and 0.1 mg/L naphthaleneacetic acid and solidified with 0.7% agar. The pH of the medium was adjusted to 5.8. 2-iP was filter-sterilized and added to the medium after autoclave.

As is evidenced in the pictures of *Figs. 13A and 13B*, explants from transgenic plants (1' and 2') regenerate shoots and roots at a higher frequency and earlier than wild type (WT), consistent with a higher meristematic competence. The evidence of difference between the transformed and wild-type cotyledons was dramatically evidenced at day 23 (*Fig. 13A*) and more so 37 days into culturing (*Fig. 13B*).

Example 18: Effect of overexpression of AVP-1 on Shoot Regeneration from Root and Cotyledon Explants in *Arabidopsis* Plants

Root and cotyledon (5 days old) explants from wild-type (WT) and transgenic (1' and 2') AVP-1 overexpressing *Arabidopsis* plants were placed in the shoot induction medium as described in Example. 16. As evidenced in *Fig. 6*, explants from the transgenic plants (1' and 2') generated new structure earlier than wild type consistent with a higher meristematic competence.

Example 19: Isolation Of Mutants In The Transporters

Genetic approaches are very powerful in analyzing complex biological traits (Serrano, R., *Crit. Rev. Plant Sci.*, 13:121-138 (1994)) Reverse genetics is a very important new tool for plant biologists. The generation of a good collection of tagged knockouts by Sussman and coworkers (Krysan, P., *et al.*, *Proc. Natl. Acad. Sci. USA*,

93:8145-8150 (1996)) has open a very important avenue for the analysis of gene disruptions in *Arabidopsis*.

The Arabidopsis Knock-out Facility of the University of Wisconsin Madison (world wide web at [biotech.wisc.edu/NewServicesAndResearch /Arabidopsis](http://biotech.wisc.edu/NewServicesAndResearch/Arabidopsis)) may be used to search among the 60,480 Arabidopsis (ecotype WS) lines that have been transformed with the T-DNA vector pD991 for the presence of T-DNA inserts within *AtCLC-c*, *AtCLC-d*, *AVP1*, *AtNHX1* and their homologues. The phenotypes of the above knock-outs will shed light towards the understanding of the physiological roles of these transporters in normal and stress conditions. An initial characterization of the knockout plants includes testing for their salt tolerance and their  $\text{Na}^+/\text{K}^+$  ratios. The generation of double knock-outs via crosses help to further understand the interaction among the transporters as well as the crosses with the *35S AVP1* and the *35S AtNHX1* transgenic plants.

To search for *Arabidopsis* knock-out PCR primers may be designed following the guidelines detailed in the University of Wisconsin web site. Tested primers may be sent to UW-Madison, where 62 PCR reactions that are sent to us for Southern blot analysis may be performed. Positive PCR products are sequenced. If the sequence reveals that there is a T-DNA inserted within the gene the gene specific primers are sent for another set of PCR reactions in order to determine which of the 9 possible pools of 225 contains the knockout. After identifying the pool of interest, 25 tubes of seeds are screened for the individual plant carrying the T-DNA knock-out.

#### Example 20: Cation Detoxification In Plant Cells

The studies described herein together with other evidence strongly indicate that yeast and plants share pathways and signals for the trafficking of vesicles from Golgi network to the vacuole (Gaxiola, R., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999); Marty, F., "The Biogenesis of Vacuoles: Insights from Microscopy. In: *The Plant Vacuole*, 1-42, Leigh, R.A. and Sanders, D., Academic Press, San Diego (1997); Bassham, D.C., *et al.*, *Plant Physiol*, 117:407-415 (1998)).

Without wishing to be bound by theory, it is believe likely by the present inventor that in both systems a pre-vacuolar compartment is a dynamic entity that detoxifies the cytoplasm from toxic cations and delivers its cargo either to the vacuole, or

directly to the cell exterior. Both the *gefl* chloride channel and *Nhx1*  $\text{Na}^+/\text{H}^+$  exchanger have been localized to the yeast pre-vacuolar compartment (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). The behavior of the *gefl*-GFP chimera in yeast cells *in vivo* have been monitored indicating that its localization varies depending  
5 the environmental conditions. Furthermore, it has been shown that two of the four *A. thaliana* CLC chloride channel genes CLC-*c* and -*d* are capable of suppressing *gefl* mutant phenotypes implying a similar localization (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)).

In order to understand how and where this cation detoxification takes place  
10 in plant cells the intracellular localization of GFP chimeras of AVP1, AtNHX1 and AtCLC-*c* and -*d* (Hong, B., *et al.*, *Plant Physiol.*, 119:1165-1175 (1999)) may be monitored *in vivo*. Confocal microscopy may be also used to address co-localization of the different transporters. For this purpose HA-tagged versions or antibodies of the transporters under study are required (Guiltinan, M.J., *et al.*, *Meth. Cell Biol.*, 49:143-151  
15 (1995); Jauh, G. -Y., *et al.*, *Plant Cell*, 11:1867-1882 (1999); Mullen, R.T., *et al.*, *Plant J.*, 12:313-322 (1997)).

For the constructions of the GFP-chimeras the soluble versions GFP with improved fluorescence in *A. thaliana* reported by Davis and Viestra (Davies, S.J., Viestra, R.D., "Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis*  
20 *thaliana*, <http://brindabella.mrc-lmb.cam.ac.uk/IndexGFP.html> (1998)). may be used. Two types of GFP-chimeras may be made, namely a set under the regulation of the native promoter and another set under the regulation of the 35S promoter. The resulting T-DNA vectors containing the GFP-chimeras are transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of  
25 *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al.*, *C.R. Jeances Acad. Sci. Ser. III Sci. Vie*, 316:1194-1199 (1993)). For the hemagglutinin (HA) epitope tagging a PCR strategy designed for yeast but modified to tag plant genes expressed in yeast vectors may be used. Futcher and coworkers designed vectors containing the URA3 yeast gene flanked by direct repeats of epitope tags (HA) (Schneider, B.L., *et al.*, *Yeast*, 11:1265-  
30 1274 (1995)). Via PCR the tag-URA3-tag cassette may be amplified such that the resulting PCR fragment possess homology at each end to the gene of interest. *In vivo* recombination in yeast can be then used to direct the integration of the PCR-chimera to the

plasmid carrying the plant ORF of interest, transformants are selected by the URA<sup>+</sup> phenotype. The URA3 gene can be "popped out" when positive transformants are grown in the presence of 5-fluoro-orotic acid. The vector carrying the plant gene has a selection marker different than the URA3 gene.

5                   In conclusion, the manipulation of vacuolar V-PPases in economically important crops could provide an important avenue for crop improvement. Drought and freeze tolerant cultivars could provide new agricultural approaches in areas lost due to drought or minimal rainfall, as well as to provide farmers with protection from unanticipated frosts (freezing rain etc.). Such crops may also be able to be raised on soils  
10                   considered too saline for wild type crops.

                  While the invention has been described with respect to preferred embodiments, those skilled in the art will readily appreciate that various changes and/or modifications can be made to the invention without departing from the spirit or scope of the invention as defined by the appended claims. All references cited in this specification  
15                   are herein incorporated by reference to the same extent as if each individual reference was specifically and individually indicated to be incorporated by reference.



What is claimed is:

1. A method of making a transgenic plant with increased meristematic activity comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant so to increase vacuolar pyrophosphatase activity, to yield transformed cells in the plant, thereby  
5 producing a transgenic plant which has increased meristematic activity.
2. The method of Claim 1 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which has increased meristematic activity, thereby producing a transgenic plant with improved meristematic activity.
3. The method of Claim 1 wherein the exogenous nucleic acid encodes AVP1 or homologue thereof.
4. The method of Claim 3 wherein the AVP1 or AVP1 homologue is present in a construct designed to overexpress AVP1.
5. The method of Claim 4 wherein the construct comprises the AVP1 gene or AVP1 homologue is operably linked to a chimeric promoter designed to overexpress AVP1 or homologue thereof.
6. The method of Claim 5 wherein the AVP1 gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of: tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.
7. The method of Claim 5 wherein the AVP1 gene or AVP1 homologue is operably linked to a double tandem enhancer of a 35S promoter.
8. The method of Claim 3 wherein the AVP1, or homologue thereof, is derived from a wild type plant.
9. The method of Claim 3 wherein the AVP1, or homologue thereof, is derived from a transgenic plant.

10. The method of Claim 3 wherein the AVP1, or homologue thereof, is derived from a mutant plant.
11. The method of Claim 3 wherein the AVP1, or homologue thereof, is synthetically derived.
12. The method of Claim 3 wherein the AVP1, or homologue thereof, is naturally and synthetically derived.
13. A transgenic plant produced by the method of Claim 1.
14. A method of making a transgenic plant with increased meristematic competence comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant to yield transformed cells in the plant, thereby producing a transgenic plant which has increased meristematic competence.
15. The method of Claim 14 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which has increased meristematic activity, thereby producing a transgenic plant with improved meristematic activity.
16. The method of Claim 14 wherein the exogenous nucleic acid encodes AVP1 or homologue thereof.
17. The method of Claim 16 wherein the AVP1 or AVP1 homologue is present in a construct designed to overexpress AVP1.
18. The method of Claim 17 wherein the construct comprises the AVP1 gene or AVP1 homologue is operably linked to a chimeric promoter designed to overexpress AVP1 or homologue thereof.
19. The method of Claim 18 wherein the AVP1 gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of: tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.

20. The method of Claim 18 wherein the AVP1 gene or AVP1 homologue is operably linked to a double tandem enhancer of a 35S promoter.
21. The method of Claim 16 wherein the AVP1, or homologue thereof, is derived from a wild type plant.
22. The method of Claim 16 wherein the AVP1, or homologue thereof, is derived from a transgenic plant.
23. The method of Claim 16 wherein the AVP1, or homologue thereof, is derived from a mutant plant.
24. The method of Claim 16 wherein the AVP1, or homologue thereof, is synthetically derived.
25. The method of Claim 16 wherein the AVP1, or homologue thereof, is naturally and synthetically derived.
26. A method of making a transgenic plant with increased biomass comprising introducing into one or more cells of a plant a nucleic acid construct which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the cell to yield transformed cells thereby producing a transgenic plant with increased biomass.
27. The method of Claim 26 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant with increased biomass.
28. A transgenic plant produced by the method of Claim 26
29. A method of making a transgenic plant with an increased biomass over its corresponding wild type plant, wherein the increased biomass relates to an increase in the biomass of a plant part selected from the group consisting of: leaves, stems, roots, seeds, flowers, and fruits, said method comprising introducing into one or more cells of a plant, an exogenous nucleic acid which alters expression of vacuolar pyrophosphatase such so as to enhance the activity of the vacuolar

pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant with an increased biomass.

30. The method of Claim 29 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant with an increased biomass.
31. The method of Claim 29 wherein the exogenous nucleic acid encodes AVP1, or homologue thereof.
32. The method of Claim 31 wherein the AVP1, or homologue thereof, is present in a construct designed to overexpress AVP1, or homologue thereof.
33. The method of Claim 32 wherein the construct comprises the AVP1 gene, or homologue thereof, operably linked to a chimeric promoter designed to overexpress AVP1.
34. The method of Claim 33 wherein the AVP1 gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of: tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.
35. The method of Claim 32 wherein the AVP1 gene, or homologue thereof, is operably linked to a double tandem enhancer of a 35S promoter.
36. The method of Claim 31 wherein the AVP1, or homologue thereof, is derived from a wild type plant.
37. The method of Claim 31 wherein the AVP1, or homologue thereof, is derived from a transgenic plant.
38. The method of Claim 31 wherein the AVP1, or homologue thereof, is derived from a mutant plant.
39. The method of Claim 29 wherein the transgenic plant is grown in soil.
40. The method of Claim 29 wherein the transgenic plant is grown hydroponically.

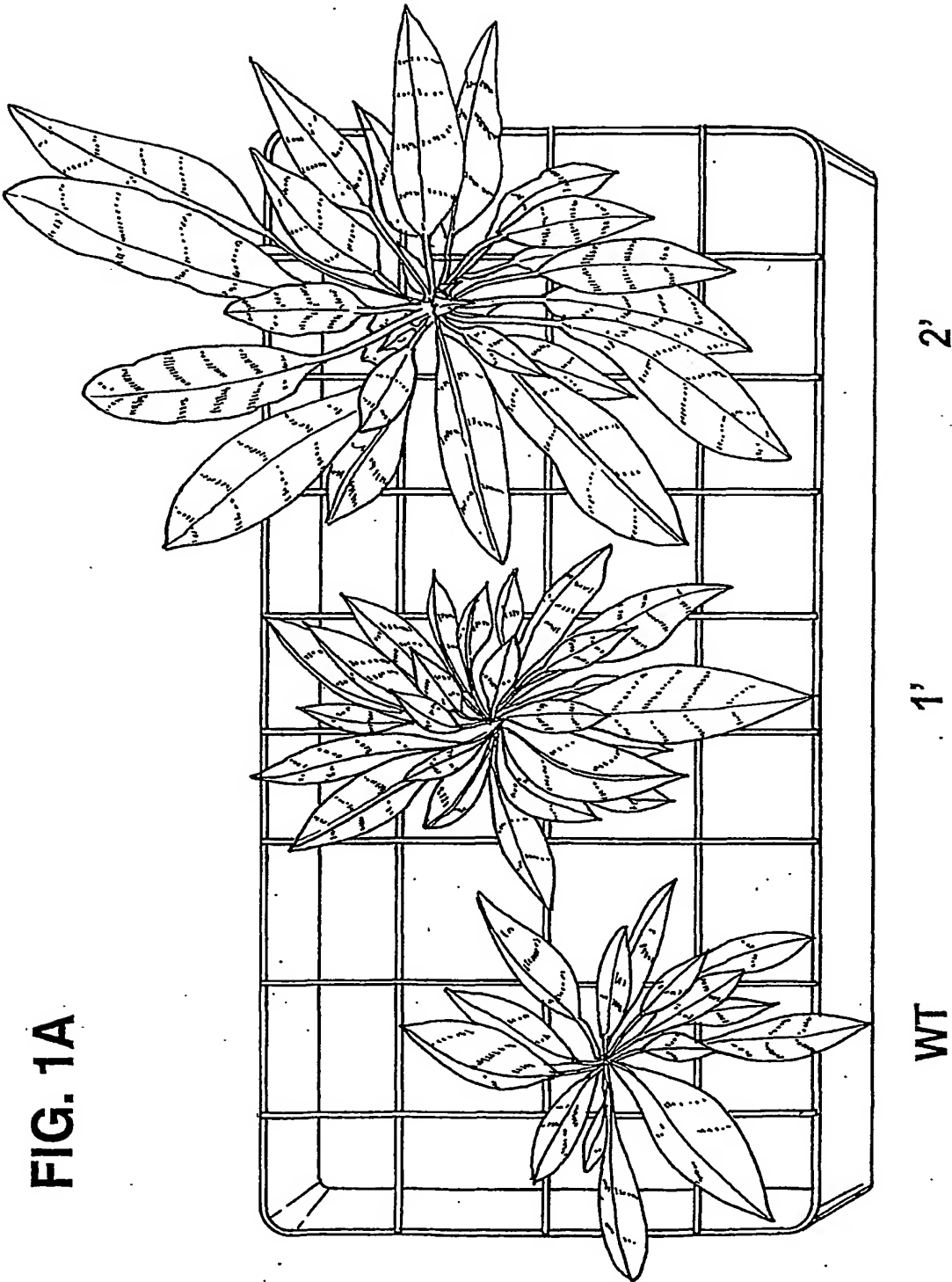
41. The method of Claim 29 wherein a cell from the transgenic plant is grown in culture.
42. A transgenic plant produced by the method of Claim 29.
43. A method of producing a transgenic plant with a stem structure thicker than wild type comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant to yield transformed cells, thereby producing a transgenic plant with a thickened stem structure.
44. The method of claim 43 wherein the stem structure is selected from group consisting of: wood, bark and cambium.
45. The method of Claim 43 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant with a thickened stem structure, thereby producing a transgenic plant with thickened stem structure.
46. A method of making a transgenic plant having increased root structure compared to its corresponding wild type plant comprising introducing into one or more cells of a exogenous nucleic acid which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant to yield transformed cells, thereby producing a transgenic plant having increased root structure.
47. The method of Claim 46 wherein the exogenous nucleic acid encodes AVP1, or homologue thereof.
48. A transgenic plant produced by the method of Claim 46.
49. A method of making a transgenic plant having increased shoot regeneration capacity as compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant

- 5 to yield transformed cells, thereby producing a transgenic plant having increased shoot regeneration capacity.
50. The method of Claim 49 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which has increased shoot regeneration capacity, thereby producing a transgenic plant with improved shoot regeneration capacity.
51. The method of Claim 49 wherein the exogenous nucleic acid encodes AVP1, or homologue thereof.
52. The method of Claim 51 wherein the AVP1, or AVP1 homologue, is present in a construct designed to overexpress AVP1.
53. The method of Claim 52 wherein the construct that comprises the AVP1 gene or AVP1 homologue is operably linked to a chimeric promoter designed to overexpress AVP1 or homologue thereof.
54. The method of Claim 53 wherein the AVP1 gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of: tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.
55. The method of Claim 53 wherein the AVP1 gene or AVP1 homologue is operably linked to a double tandem enhancer of a 35S promoter.
56. The method of Claim 52 wherein the AVP1, or homologue thereof, is derived from a wild type plant.
57. The method of Claim 52 wherein the AVP1, or homologue thereof, is derived from a transgenic plant.
58. The method of Claim 52 wherein the AVP1, or homologue thereof, is derived from a mutant plant.
59. The method of Claim 52 wherein the AVP1, or homologue thereof, is synthetically derived.

60. The method of Claim 52 wherein the AVP1, or homologue thereof, is naturally and synthetically derived.
61. A transgenic plant produced by the method of Claim 49.
62. A method of making a transgenic plant having increased root regeneration capacity as compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant to yield transformed cells, thereby producing a transgenic plant having increased root structure.
63. The method of Claim 62 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which has increased root regeneration capacity, thereby producing a transgenic plant with improved root regeneration capacity.
64. The method of Claim 62 wherein the exogenous nucleic acid encodes AVP1, or homologue thereof.
65. The method of Claim 64 wherein the AVP1, or AVP1 homologue, is present in a construct designed to overexpress AVP1.
66. The method of Claim 65 wherein the construct that comprises the AVP1 gene or AVP1 homologue is operably linked to a chimeric promoter designed to overexpress AVP1 or homologue thereof.
67. The method of Claim 66 wherein the AVP1 gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of: tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.
68. The method of Claim 64 wherein the AVP1 gene or AVP1 homologue is operably linked to a double tandem enhancer of a 35S promoter.

69. The method of Claim 64 wherein the AVP1, or homologue thereof, is derived from a wild type plant.
70. The method of Claim 64 wherein the AVP1, or homologue thereof, is derived from a transgenic plant.
71. The method of Claim 64 wherein the AVP1, or homologue thereof, is derived from a mutant plant.
72. The method of Claim 64 wherein the AVP1, or homologue thereof, is synthetically derived.
73. The method of Claim 64 wherein the AVP1, or homologue thereof, is naturally and synthetically derived.
74. A transgenic plant produced by the method of Claim 62.





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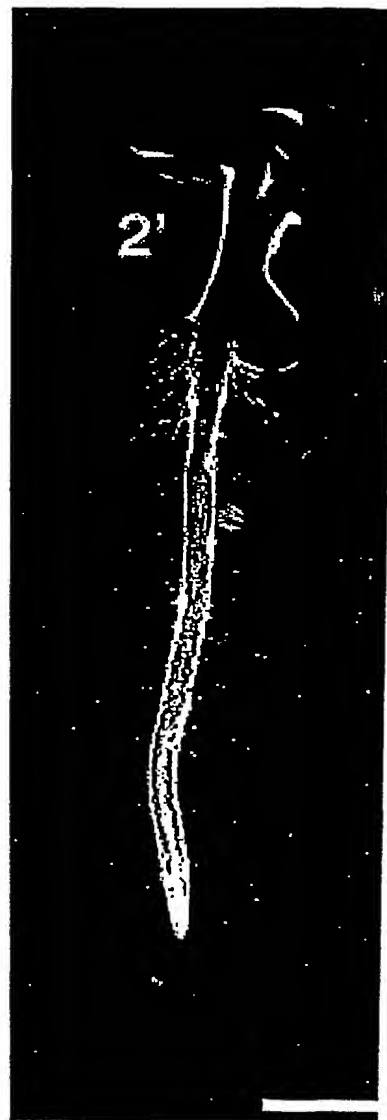


FIG. 1B(1)

FIG. 1B(2)

FIG. 1B(3)

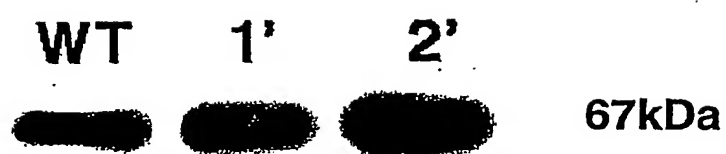
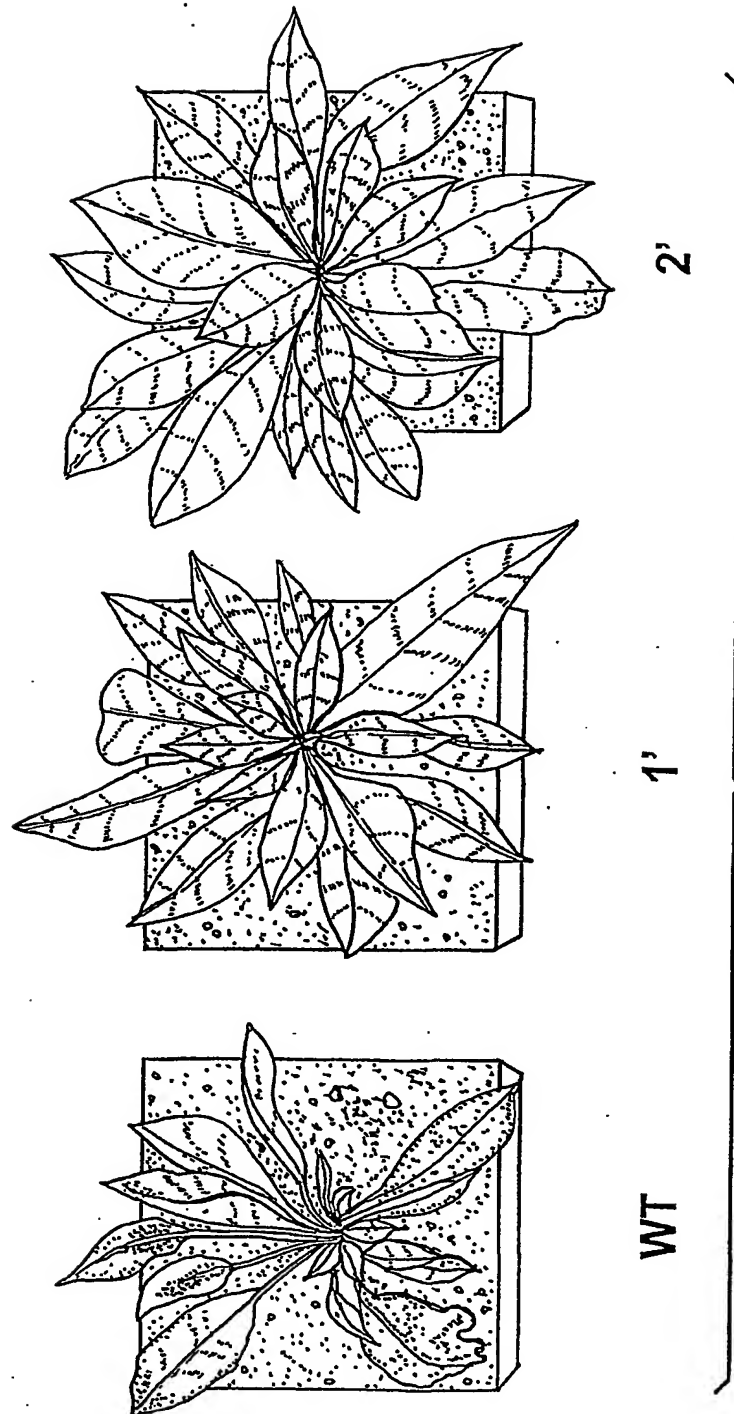


FIG. 1C

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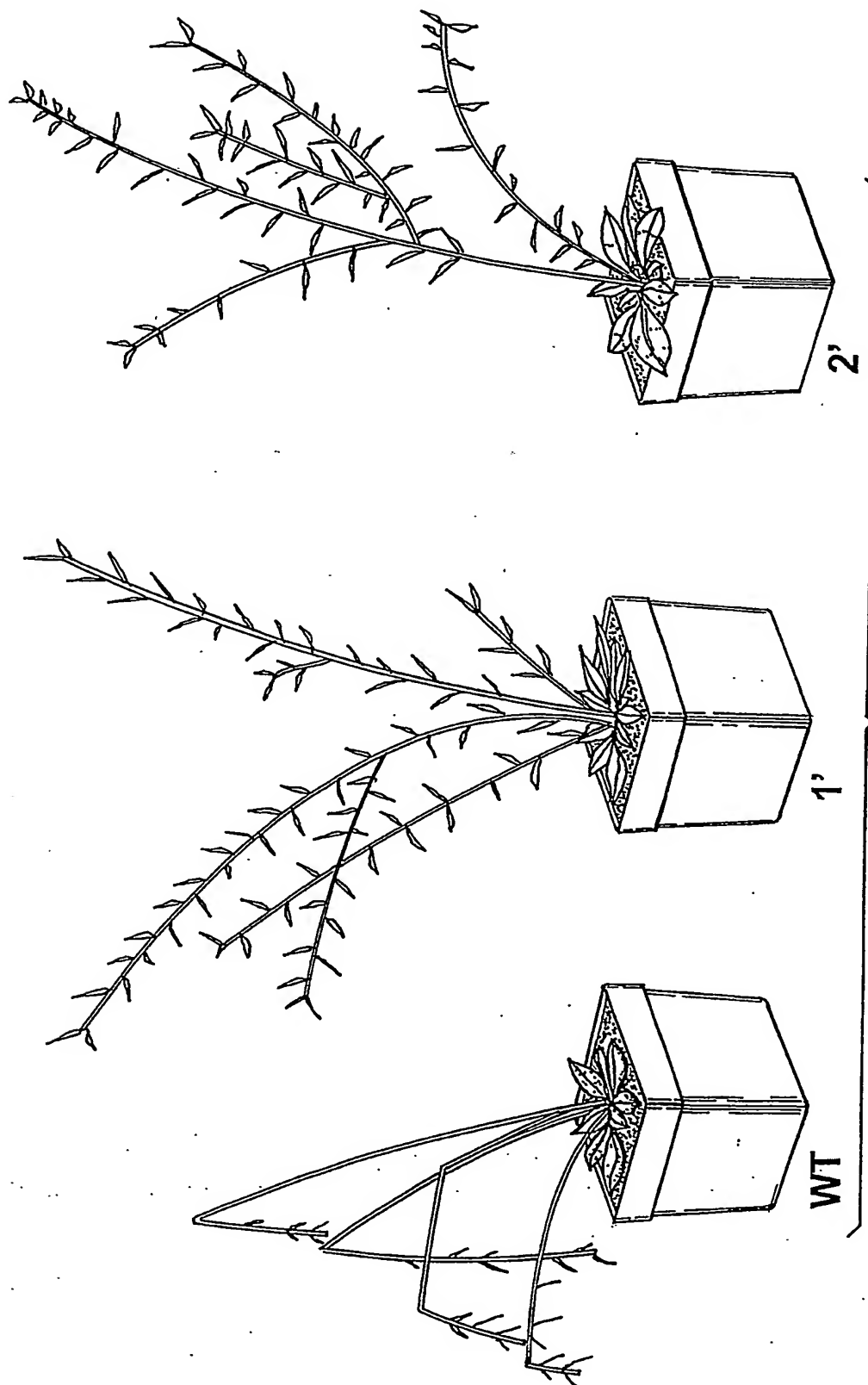


FIG. 3

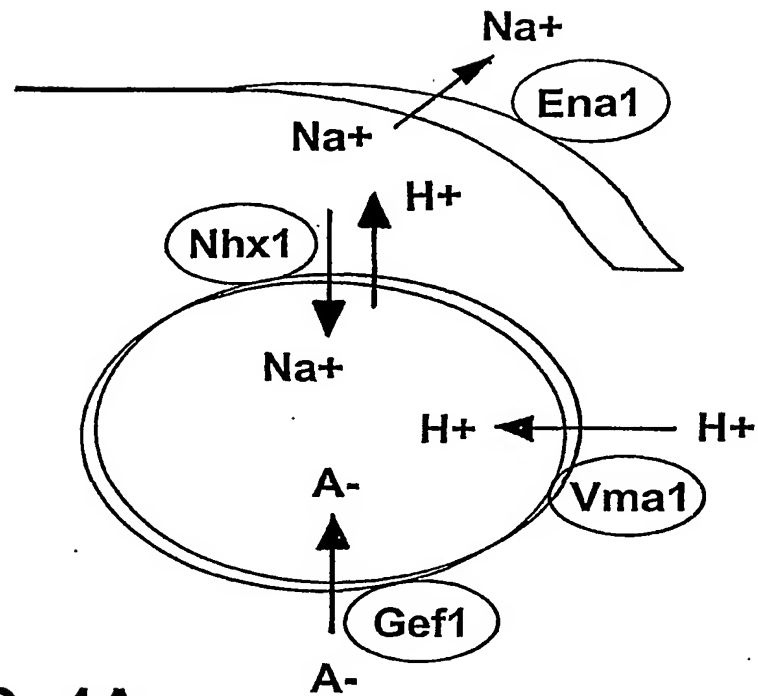


FIG. 4A

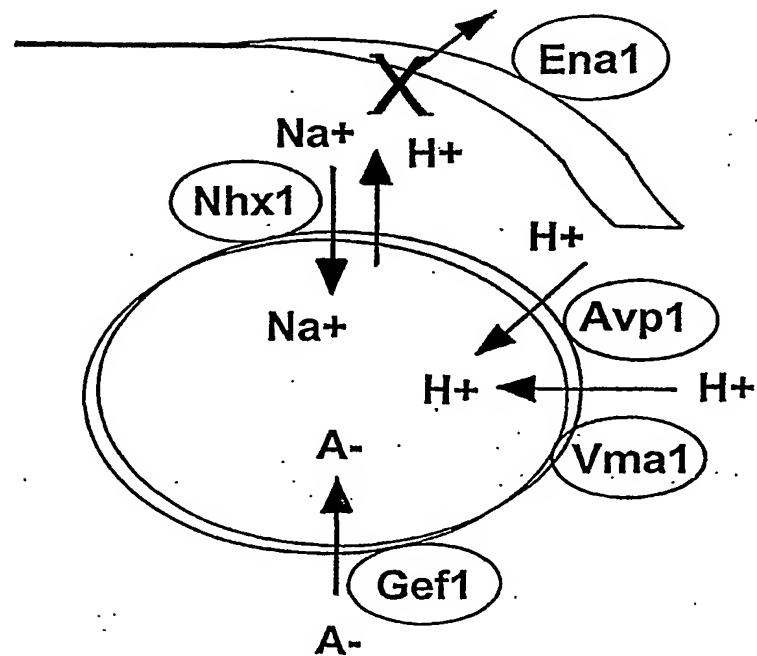


FIG. 4B

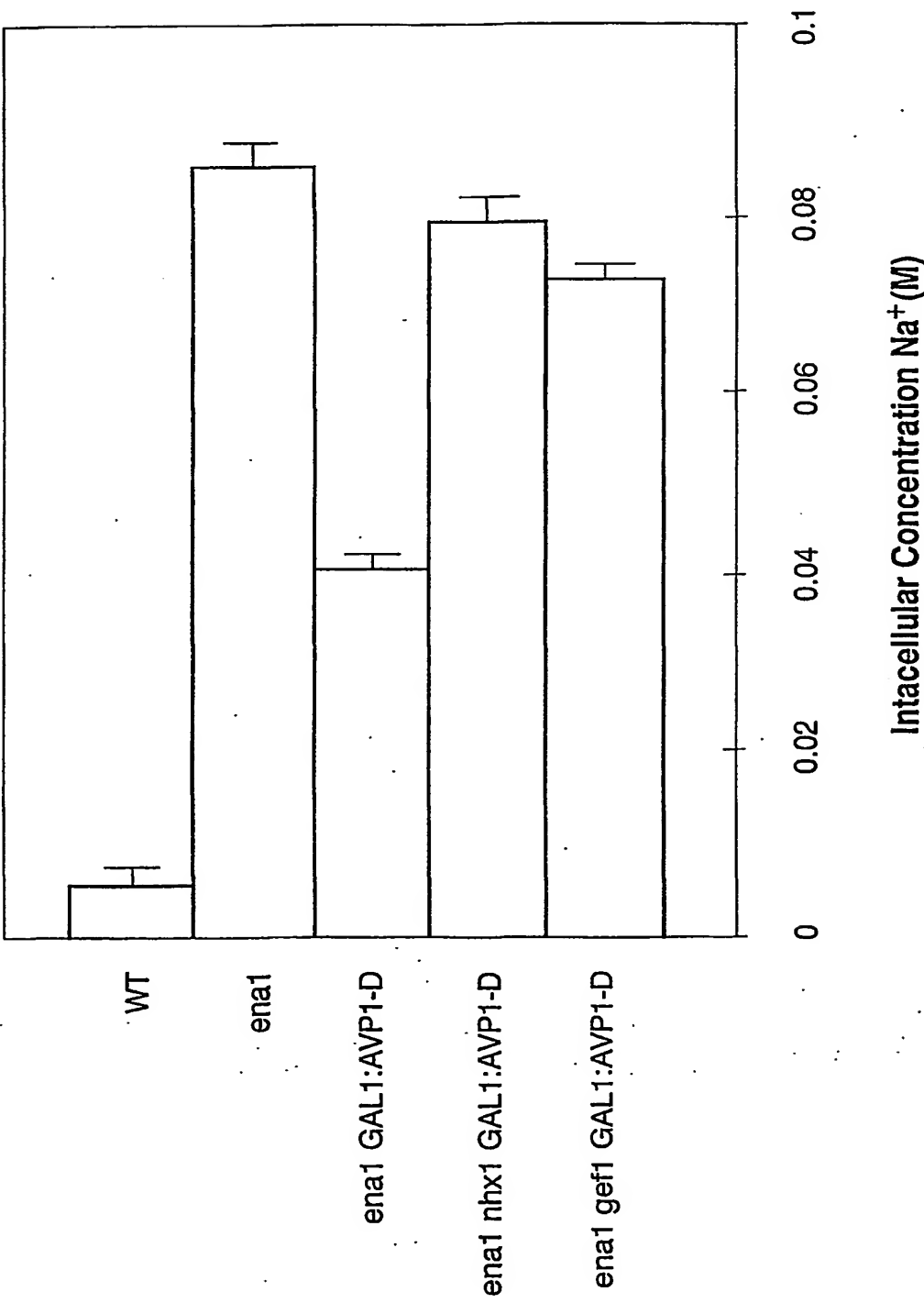
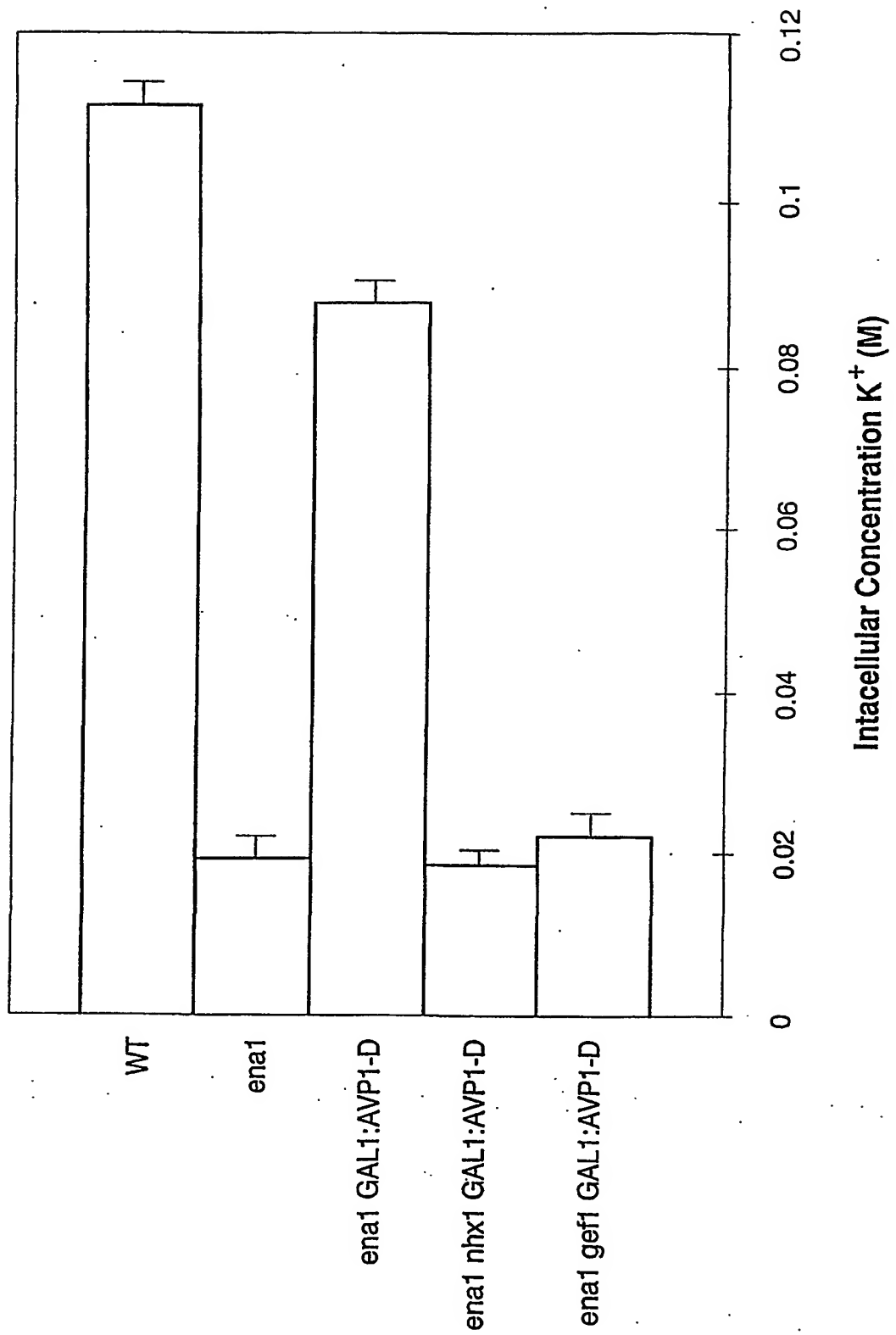


FIG. 5A

**FIG. 5B**



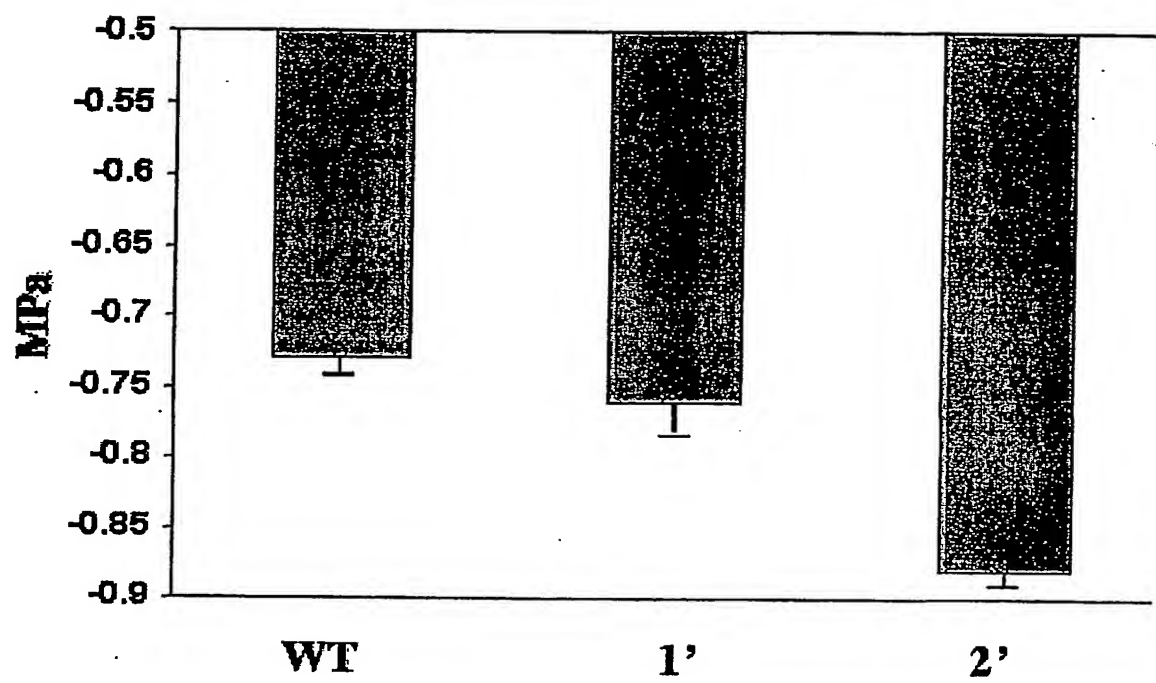
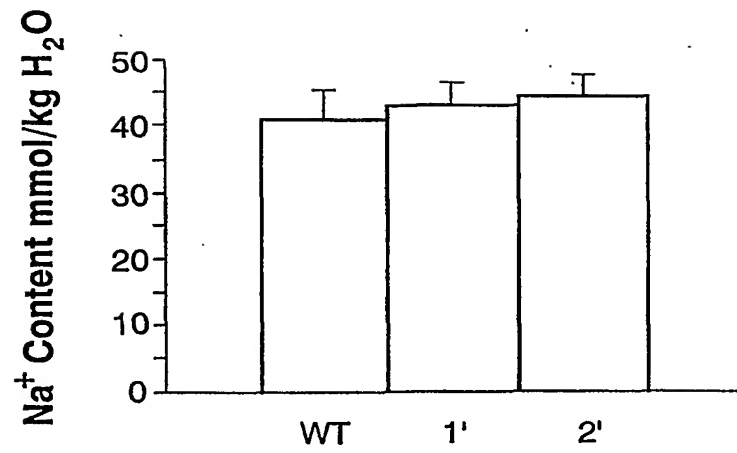
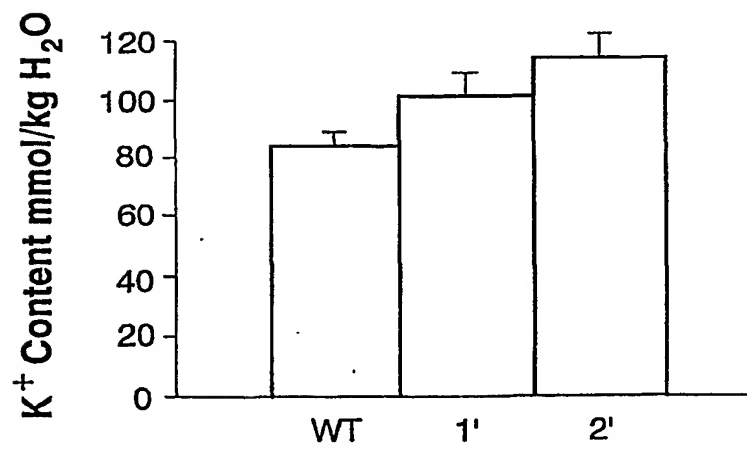
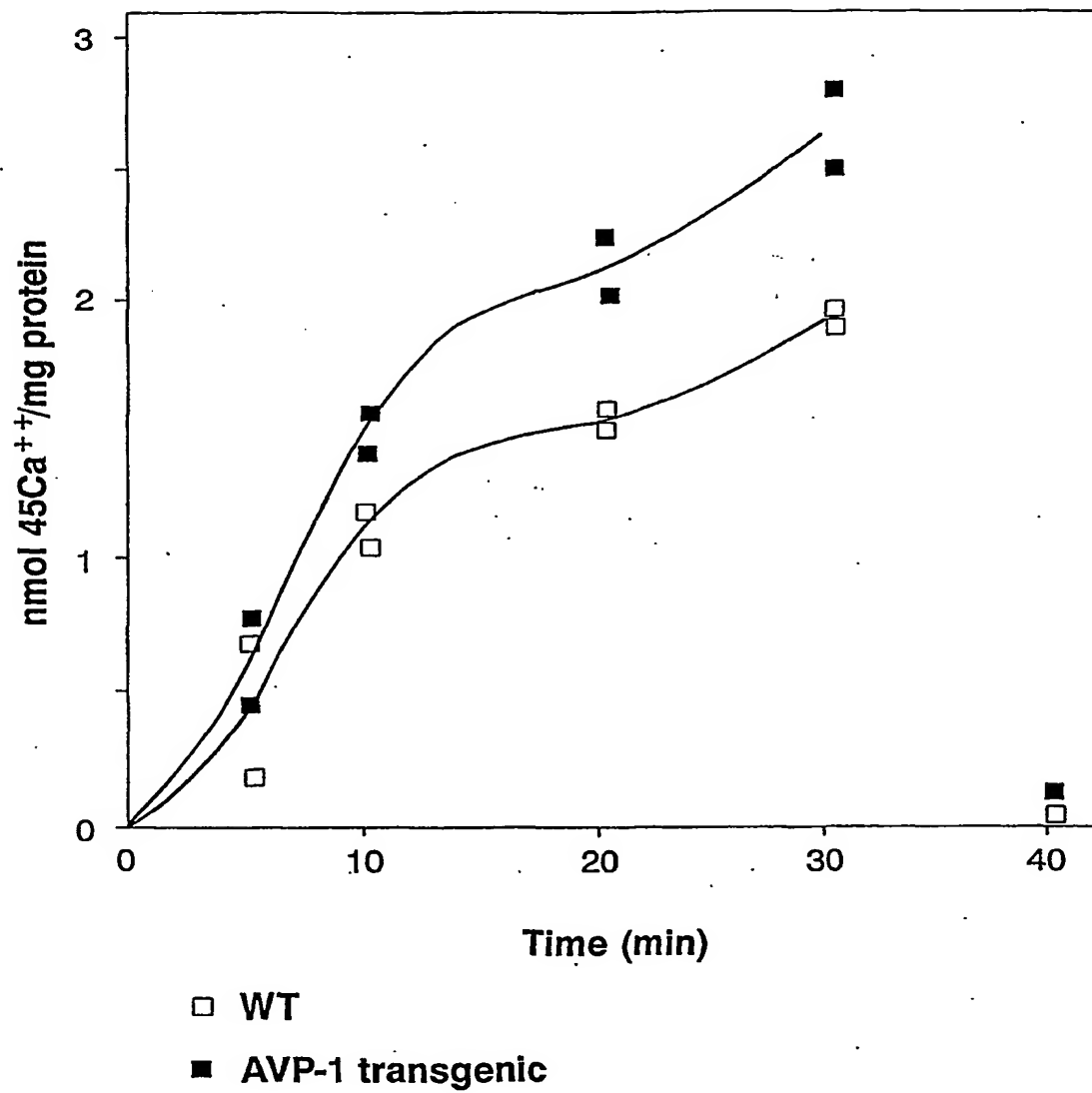
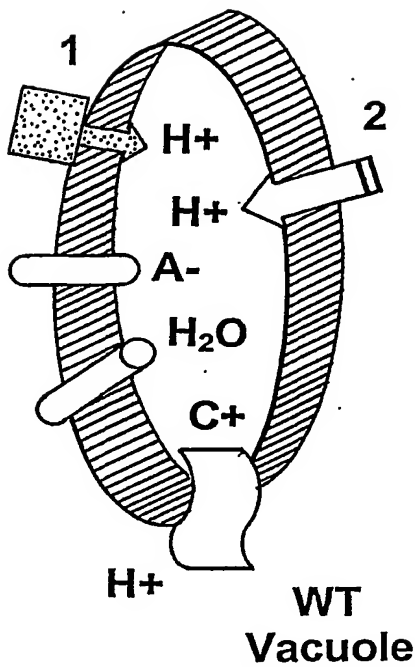
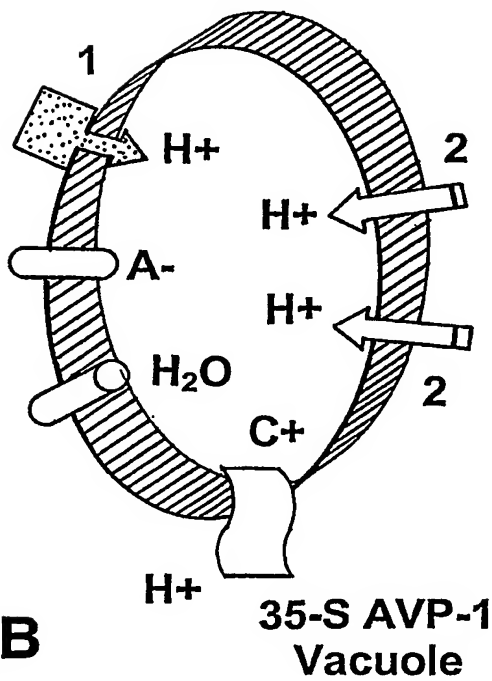


FIG. 6

**FIG. 7A****FIG. 7B**

**FIG. 8**

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**FIG. 9A****FIG. 9B**

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FIG. 10

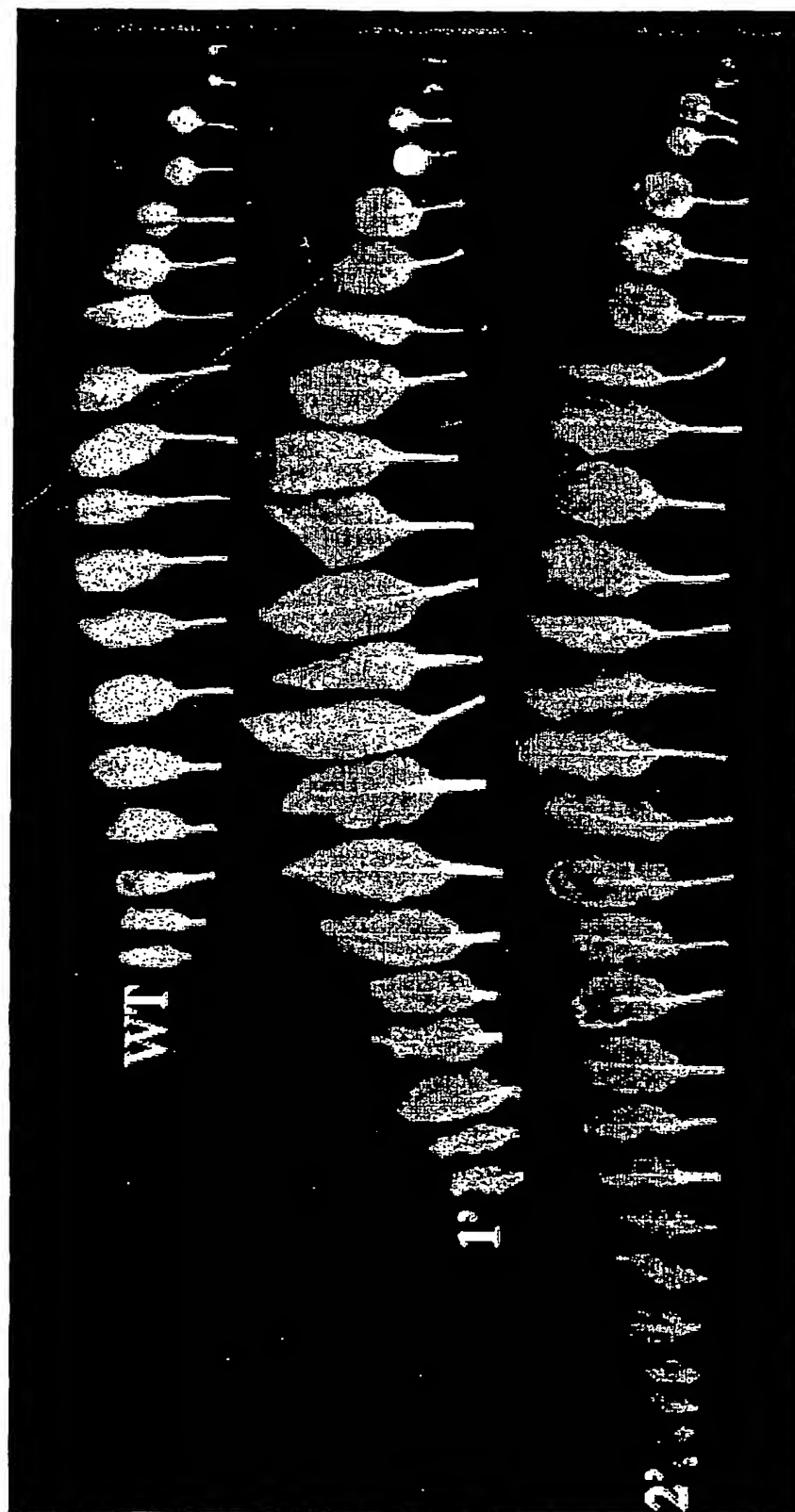
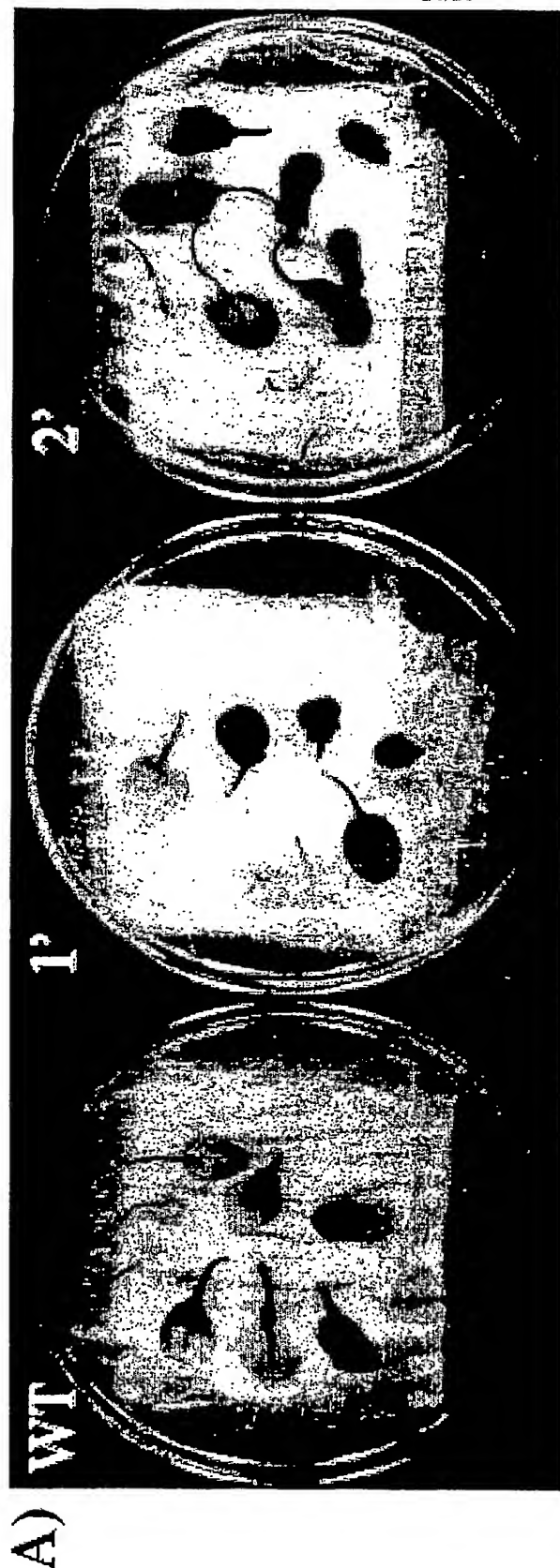


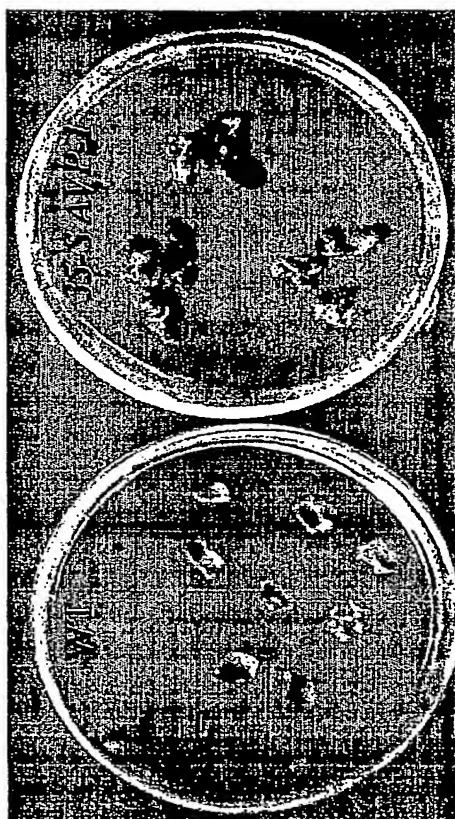
FIG. 11

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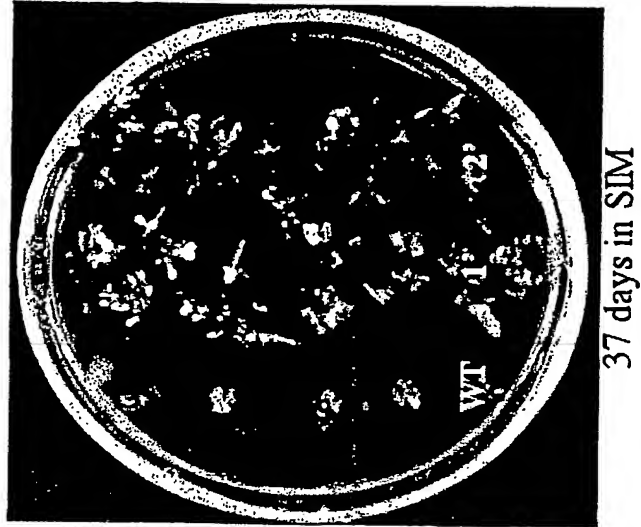
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FIG. 12



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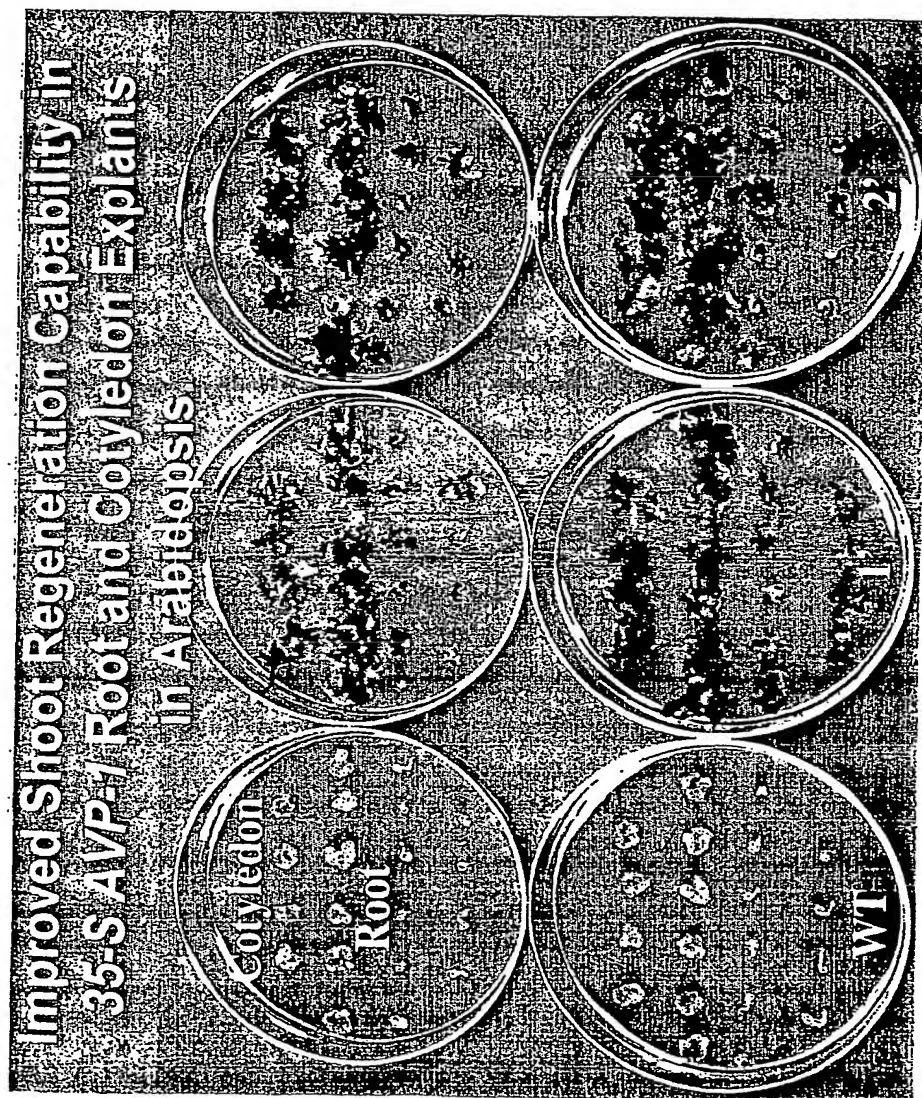
FIG. 13





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FIG. 14



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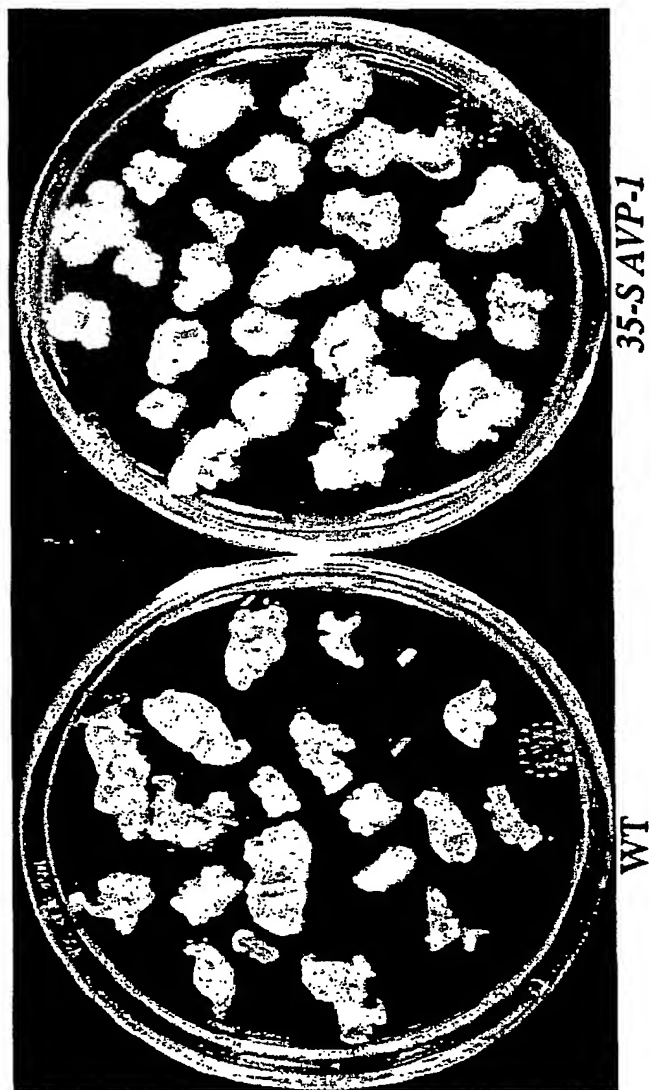


FIG. 15

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/09548

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01H 1/00, 5/00; C12N 5/04, 5/10, 15/82, 15/87

US CL : 435/ 419, 468; 800/ 290, 298

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 419, 468; 800/ 290, 298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SARAFIAN et al. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. March 1992, Vol. 89, pages 1775-1779, especially page 1777 Figure 2.	1-74
Y	GAXIOLA et al. The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proc. Natl. Acad. Sci. USA. February 1999, Vol. 96, pages 1480-1485, especially page 1481 Figure 1.	1-74

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
* "A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 May 2001 (31.05.2001)

Date of mailing of the international search report

31 JUL 2001

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Cynthia Collins

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/09548

**Continuation of B. FIELDS SEARCHED Item 3:** WEST, STN (medline, agricola, caba, caplus, biosis, biotechno, embase, esbiobase); **TERMS:** inventor name, AVP1, vacuolar pyrophosphatase, gene, transgenic plant